UNIVERSITY OF ALBERTA

# Beta-Adrenergic Receptors in Tissues of Sheep: Effects of Temperature and Plane of Nutrition.

by

**Enefiok David Ekpe** ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

**Animal Science**

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **BETA-ADRENERGIC RECEPTORS IN TISSUES OF SHEEP: EFFECTS OF TEMPERATURE AND PLANE OF NUTRITION** submitted by **ENEFIOK DAVID EKPE** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL SCIENCE**.

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Dated: Nov. 18, 1997

DEDICATION

To

my ever beloved wife, **Anwangabasi Enefiok Ekpe**

and

my children, **Ukobong**, **Abasifreke** and **Etoroabasi Enefiok Ekpe**

for their invaluable support

And

To the memory of my beloved

father, **Mr. David Akpan Etighe Ekpe**

and

sister, **Miss Glory David Ekpe**

who taught me never to give up

## ABSTRACT

The aims of this thesis were to investigate the expression of beta-adrenoceptors and to determine the effects of temperature and feed intake on expression of these receptors. Twenty four Suffolk-cross wether lambs were used to determine expression of β-adrenoceptors and effects of temperature (cold, $0 \pm 2$ °C versus warm, $23 \pm 2$ °C) and feed intake (restricted versus *ad libitum*) on densities and binding affinities. Metabolic and endocrine responses to cold exposure and feed restriction were investigated in these animals to assess adrenoceptor responses. Cold exposure increased metabolic rate, plasma concentrations of insulin, triiodothyronine and cortisol, and reduced rectal temperatures. Feed restriction increased plasma cortisol concentration, and reduced average daily gain, metabolic rate, plasma concentrations of insulin and triiodothyronine.

Radioligand binding studies, using [$^3$H]dihydroalprenolol, indicated that β1- and β2-adrenoceptors coexist in *biceps femoris*, *semitendinosus* and *gastrocnemius* muscles. The density of β1-adrenoceptors was more than that of β2-adrenoceptors, with β1-adrenoceptors being about 56 - 87 % of the total. Cold exposure increased density of β1-adrenoceptors in animals with restricted feed. Feed restriction increased density of β1-adrenoceptors in cold and warm environments. There was no effect on receptor binding affinity or β2-adrenoceptors.

Radioligand binding studies also indicated coexistence of β1- and β2- adrenoceptors in heart, kidney and liver. The density of β1-adrenoceptors (heart, 88 - 95 %; kidney, 86 %; liver, 79 %) was more than that of β2-adrenoceptors. Cold exposure and feed

restriction reduced (in heart) and increased (in kidney and liver) β1-adrenoceptor density. There was no effect on density of β2-adrenoceptors, but cold temperature and feed restriction reduced receptor binding affinities in kidney and liver. There was no negative co-operativity or presence of multiple binding sites for the binding of dihydroalprenolol.

A molecular cloning technique was used to determine expression of β-adrenoceptors in ovine tissues. Northern blotting indicated one predominant β1-adrenoceptor messenger ribonucleic acid (BAR-1 mRNA) species with a size of about 2.2 Kb recognized by human BAR-1 cDNA probe. Cold temperature and feed restriction increased BAR-1 mRNA levels in *biceps femoris*, *semitendinosus*, kidney and liver. Feed restriction reduced BAR-1 mRNA levels in heart. An human BAR-2 probe did not detect any BAR-2 mRNA transcript, probably due to its low abundance.

These results indicate that β-adrenoceptors are expressed in ovine tissues. Through their effects on β1-adrenoceptors, cold exposure and feed restriction could reduce (in heart) and increase (in other tissues) metabolic responsiveness of tissues to catecholamines. These effects could be at the transcriptional or post-transcriptional level, and this may be related to alterations in hormonal concentrations in blood plasma.

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## List of Abbreviations

| Abbreviation | Meaning |
|---|---|
| ADG | Average daily gain |
| Adrenoceptors | Adrenergic receptors |
| ATP | Adenosine triphosphate |
| BAR | Beta-adrenergic receptor |
| Bmax | Maximum binding capacity (receptor density) |
| β | Beta (β1 = beta-1; β2 = beta-2; β3 = beta-3; β-AR = beta-adrenergic receptor) |
| CA | Cold - *ad libitum* |
| cAMP | Cyclic adenosine monophosphate |
| Ci | Curie |
| cm | Centimeter |
| CR | Cold - restricted |
| °C | degree centigrade |
| d | Day |
| DHA | Dihydroalprenolol ([$^3$H]DHA = tritiated dihydroalprenolol) |
| DM | Dry matter |
| DNA | Deoxyribonucleic acid (cDNA = complementary deoxyribonucleic acid) |
| EDTA | Ethylenediamine tetra-acetic acid |
| FE | Feed efficiency |
| fmol | Femptomole |
| h | Hour |

| | |
|---|---|
| Kb | Kilo-base pairs |
| KCl | Potassium chloride |
| Kd | Equilibrium dissociation constant (receptor binding affinity) |
| L | Liter (mL = milliliter; μL = microliter) |
| M | Mole (mM = millimole; nM = nanomole; μM = micromole) |
| ME | Metabolizable energy |
| mg | Milligramme |
| min | Minute |
| MJ | Megajoule |
| NRC | National Research Council |
| PMSF | Phenylmethyl-sulfonyl fluoride |
| RNA | Ribonucleic acid (mRNA = messenger ribonucleic acid) |
| rpm | Revolution per minute |
| T3 | Triiodothyronine |
| T4 | Tetraiodothyronine (thyroxine) |
| WA | Warm - *ad libitum* |
| WR | Warm - restricted |
| α | Alpha (α1 = alpha-1; α2 = alpha-2; α-AR = alpha adrenergic receptor) |

# CHAPTER ONE

## 1.1 INTRODUCTION

The ability to promote and control growth, and to improve efficiency of milk and meat production, is a major goal in livestock production and has a tremendous economic potential wherever animal husbandry is practised. Most aspects of livestock production are based on the hormonal control of energy and protein metabolism. Therefore, studies involving experimental manipulation of energy and protein metabolism using hormones have become increasingly popular because of their economic potential in livestock production.

Endogenous catecholamines are major regulators of energy and protein metabolism. The physiologically relevant catecholamines, epinephrine (E), norepinephrine (NE) and dopamine act in concert with other key endocrine regulators of growth such as insulin, growth hormone and insulin-like growth factor-1. Despite their structural simplicity, catecholamines induce responses as diverse as glycogenolysis, aggregation of platelets, contraction of the myometrium, increased contractility of the heart, and lipolysis in adipose tissue (Williams and Lefkowitz 1978, Hadley 1984, Fillenz 1990). In ruminants, adrenergic agents, through their effects on energy and protein metabolism, are associated with altered milk production and quality, increased lean meat production and increased adaptability of animals to their environments (Jaster and Wegner 1981, Kim et al 1992). By lowering maintenance energy requirements and conserving energy, catecholamines also help to reduce the cost of animal production (Hunter 1992, Hunter et al. 1993). In

addition, E and NE also act to effect (or modulate) the secretion and / or actions of various neurotransmitters, neurohormones and hormones in various tissues in animals (Hadley 1984).

Research has shown that E and NE initiate biological responses by first interacting with discrete sites (receptors) on the cell membranes. These adrenergic receptors were originally classified as alpha- and beta- adrenergic receptors on the basis of different responses to E, NE and a synthetic agent, isopropyl-noradrenaline (Ahlquist 1948). Through the use of an extended range of sympathomimetic agents and antagonists, these receptors have been subdivided based on their differences in pharmacological profiles (see section 1.3). The main focus of this thesis is to study the expression of beta-adrenergic receptors in different tissues of sheep in relation to induced changes in metabolism.

Three methods have been used to study adrenergic receptors (see Williams and Lefkowitz 1978 for a review). The first method is the functional response assay, which includes quantification of biological responses and abilities to stimulate membrane bound adenylate cyclase and release cyclic adenosine monophosphate (cAMP). The second method, a radioligand binding technique, is a direct method performed by incubating a membrane preparation or dispersed cell suspension with a radioligand until a steady state level of binding is attained. The radioligand bound to sites in the membranes is then isolated by either centrifugation or vacuum filtration through glass fibre filters, followed by a wash of ice-cold buffer. Specific binding is determined by subtracting non-specific binding from total binding value. The non-specific binding includes the amount of

labeled ligand trapped in the pellets or non-specifically bound to non-adrenergic receptors, and is determined by measuring the amount of radioactivity bound in the presence of high concentrations of unlabeled adrenergic ligand (which should occupy all the true receptor sites). The temperature and time of incubation, and the choice of radioligand can influence the characterization of the adrenergic receptors being studied (Mersmann and McNeel 1992). The third method of receptor study involves analysis of the expression of receptor messenger ribonucleic acids (mRNA), using recombinant deoxyribonucleic acid (DNA) techniques - the quantification of the receptor mRNA gives a measure of the receptor gene expression and is normally positively related to the number of receptors (Arner et al. 1990).

The objectives of this study are to characterize the different subtypes of beta-adrenergic receptors in tissues of wethers, using radioligand binding and recombinant DNA techniques, and to determine whether the expression of these receptors are affected by cold temperature and feed restriction. The ultimate goal of characterization of these receptors is to gain insight into molecular mechanisms by which adrenergic agents elicit physiological responses. This work has considerable bearing on the energy requirements of meat and milk producing animals and on their ability to cope with environmental stresses encountered under normal production and livestock management systems. The research, therefore, has relevance for the welfare and management of livestock which are important concerns of both the livestock industry and the public in general.

However, it is important to understand certain concepts and the system (sympatho-adrenal medullary system) of which beta-adrenoceptors form a component, in order to

appreciate the relation of this study to broader aspects of metabolic adjustments and the endocrine status of the ruminant.

## 1.2 THE SYMPATHO-ADRENAL MEDULLARY SYSTEM

The sympatho-adrenal medullary system consists of two components - the sympathetic nervous system and the adrenal medulla (Hadley 1984). The sympathetic nervous system and parasympathetic nervous system form the autonomic nervous system, and their nerves are composed of preganglionic and postganglionic neurons. Their nerves supply the skin and all visceral organs (heart, kidneys, blood vessels, pancreas, adrenals, gastrointestinal tract, etc.). These nerves are mainly vasomotor and secretomotor in function (Ruffolo 1994), because their neurons innervate smooth muscles of blood vessels, exocrine glands and endocrine glands. The adrenal medulla, derived from the embryonic neural crest, is one of the two distinct cellular types of adrenal gland (Hadley 1984). Its cells are referred to as chromaffin cells due to observation that the pheochromocytes become brown when placed in contact with oxidizing agents such as chromate. In most mammals, the chromaffin tissue is surrounded by an outer adrenal cortex composed of steroid-producing cells.

The sympathetic nerves and the adrenal medulla, though integral structural and functional components of sympatho-adrenal medullary system, have been recognized as distinct neuroendocrine units (Blaak et al. 1993). Both act via the sympatho-adrenoceptor system which mediates the numerous and diverse metabolic and neuroendocrine actions of catecholamines produced in the central nervous system, in peripheral sympathetic

nerves, or in the adrenal medulla. The endogenous catecholamines that exert their physiological effects by binding to the adrenergic receptors include epinephrine, which functions as a circulating hormone released from the adrenal medulla, and norepinephrine, which serves as a neurotransmitter within the central nervous system or at the peripheral, postsynaptic nerve endings (Fillenz 1990, Christopherson et al. 1995).

The unique role of the sympatho-adrenal medullary system is to maintain the constancy of the internal environment of the body. Any decrease in blood pressure, blood glucose level, or oxygen availability leads to an acute enhancement of the sympatho-adrenal activity with resulting elevation in plasma catecholamines (Hadley 1984). Generally, this system plays an important role in helping animals to cope with stress resulting from events of external and internal origin, real or imagined, that tend to affect the homeostatic state. This system is stimulatory to hepatic glycogenolysis and adipose tissue lipolysis (Arch and Kaumann 1993) and inhibitory to lipogenesis (Stoffel and Meyer 1993), thus supplying metabolic substrates for energy release within critical tissues such as heart, brain and skeletal muscles. The net result is repartitioning of energy from fat toward the support of other tissues (Baker et al. 1984, Jones et al. 1985, Dalrymple and Ingle 1988, Choo et al. 1992).

The sympatho-adrenal medullary system is also involved in cardiovascular functions - playing important roles in the control of blood pressure, myocardial contractile rate and force, and airway reactivity. For example, red blood cells are mobilized to increase oxygen-carrying capacity of the blood in times of stress (Graham and Christopherson 1981). Thermoregulatory non-shivering thermogenesis and diet-induced thermogenesis

may result in part from sympatho-adrenal medulla activation of the brown adipose tissue (Rothwell and Stock 1979, Trayhurn 1990). In addition, this system is inhibitory to secretion of some hormones, for example, insulin, and stimulatory to release of some hormones, for example, glucagon (Hadley 1984).

The activity of the sympatho-adrenal medullary system is affected by a number of factors - endogenous or external factors, such as age, growth potential, nutrition, environmental adaptation, reproductive state and lactation. In rats, fasting suppresses and overfeeding stimulates the sympathetic nervous system (Landsberg and Young 1978, Shetty and Kurpad 1990). The suppression of activity during fasting conserves energy by reducing metabolism and heat production, whereas feeding increases activity to expend the excess calories. High protein meals stimulate the sympatho-adrenal system (Sports Nutr. News 1985). Cold exposure induces an increase in heat production, which is associated with increased catecholamine secretion due to increased activation of the sympatho-adrenal medullary system (Thompson et al. 1975, Schaefer et al. 1982, McBride and Christopherson 1984, Sasaki and Weekes 1984, Young 1989).

## 1.3 ADRENERGIC RECEPTORS

Adrenergic receptors or adrenoceptors mediate the central and peripheral actions of the primary sympathetic neurotransmitter, norepinephrine, NE (noradrenaline), and the primary adrenal medullary hormone (and central neurotransmitter), epinephrine, E (adrenaline) (Bylund et al. 1994). These adrenoceptors are found in nearly all peripheral tissues and on many neuronal populations within the central nervous system. This

ubiquitous location of these catecholamine receptors presents a challenging area of studies which have been of major interest for many years. Adrenoceptors have divergent affinity for many synthetic drugs which have proven useful in a variety of diseases, involving all of the major organ systems, for examples, hypertension, angina pectoris, congestive heart failure, cardiac arrhythmia, asthma, depression, prostatic hypertrophy and glaucoma (Bylund 1994).

Adrenoceptors were originally classified by Ahlquist (1948) as alpha- and beta-adrenoceptors on the basis of different responses to E, NE and synthetic isoproterenol (ISO), with a potency ranking of E > NE > ISO for the alpha-adrenoceptors, and potency ranking of ISO > E > NE for beta-adrenoceptors. Using an extended range of sympathomimetic agents and antagonists, these receptors have been further subdivided based on their differences in pharmacological profiles (see sections 1.3.1 and 1.3.2).

Since the discovery of the different subtypes of adrenoceptors, researchers have tried to separate the effects of stimulation of each of these receptors. Their diverse effects on all body tissues makes studying the role of catecholamines in controlling body metabolism a challenging area. By interacting with these receptors, catecholamines play important roles in the control of blood pressure, myocardial contractile rate and force, airway reactivity, and a variety of metabolic functions. In some tissues, both alpha- and beta- adrenoceptors co-exist, and in most cases, they perform opposing functions, for example, in smooth muscle, E causes contraction by acting on alpha-adrenoceptors and causes relaxation by acting on beta-adrenoceptors (Hadley 1984). This allows for tight regulation of tissue functions and rapid reaction to extracellular stimuli. In some other

tissues or cells, there is a predominance of one receptor subtype with a very small amount or absence of other receptor subtypes (Swenson 1984). The alpha-adrenoceptors tend to have more of an effect on physical attributes such as blood pressure and glandular secretion, as well as presynaptic negative feedback on NE release (McDowell and Annison 1991), although beta-adrenoceptors also affect these functions, but usually in opposition to the alpha-adrenoceptor effects. Characteristically, all adrenoceptors have seven transmembrane domains, and they belong to a family of guanine nucleotide-regulatory protein (G-protein)-coupled receptors (Jacobs 1994).

### 1.3.1 Alpha ($\alpha$)- Adrenoceptors

Langer (1974) suggested the designation of $\alpha_2$ and $\alpha_1$ for pre- and post- junctional $\alpha$-adrenoceptors, respectively, due to their different pharmacological characteristics. Studies of the interactions of agonists and antagonists with these $\alpha$-adrenoceptors extended this subclassification scheme to a functional subdivision, as opposed to an anatomical subdivision, of $\alpha$-adrenoceptors into $\alpha_1$ and $\alpha_2$ subtypes. Alpha-1 adrenoceptors have predominant effects on vasoconstriction and glycogenolysis while $\alpha_2$-adrenoceptors have predominant effects on inhibition of NE release and lipolysis (Brodde 1990, King et al. 1992).

Radioligand binding and molecular studies have identified several $\alpha_1$-adrenoceptor subtypes all having similar high affinity for prazosin, but varying affinity for the other $\alpha$-adrenoceptor antagonists. Three $\alpha_1$ -adrenoceptor cDNAs have been isolated and the receptor proteins expressed. Although the relationship of the recombinant subtypes to

those in native tissues is still unclear, the data suggest the possible existence of four subtypes, which have been designated $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$ and $\alpha_{1D}$ (Bylund et al. 1994). All of the $\alpha_1$-adrenoceptor subtypes are activated by E and NE, and there is no evidence for selective affinity of either of these catecholamines for any of the $\alpha_1$-adrenoceptor subtypes identified to date. All $\alpha_1$-adrenoceptors are positively coupled to phospholipase C through $G_q$-proteins, which stimulates hydrolysis of phosphatidyl-inositol bisphosphate (PIP2) into inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG) (Fillenz 1990). IP3 mobilizes calcium from endoplasmic reticulum to cytosol and also activates calcium channels. DAG and calcium activate protein kinase C activities. Alpha-1 adrenoceptors are also directly linked to ion channels, for examples, calcium and potassium channels.

As for $\alpha_1$-adrenoceptors, $\alpha_2$-adrenoceptors have been subdivided based on functional and radioligand binding studies, and several distinct $\alpha_2$-adrenoceptor proteins have been cloned and expressed. Pharmacological data suggest existence of multiple subtypes of $\alpha_2$-adrenoceptors, designated $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$ and $\alpha_{2D}$ (Petrash and Bylund 1986). All known $\alpha_2$-adrenoceptor subtypes can be activated by E and NE with no significant selectivity between any of them (Bylund et al. 1994). They are negatively coupled to adenylate cyclase through $G_i$-proteins in most tissues (Fillenz 1990), and are directly linked to ion channels, for example, sodium-hydrogen exchange system in platelets (Ruffolo 1994) whose stimulation leads to an influx of sodium and an efflux of hydrogen ions, intracellular alkalinization, elevated intracellular calcium, activation of membrane bound phospholipase $A_2$, release of arachidonic acid, and enzymatic conversion to thromboxane $A_2$ to trigger platelet aggregation.

### 1.3.2 Beta ($\beta$) -Adrenoceptors

Beta-adrenoceptors were initially subclassified into $\beta$1 -and $\beta$2- adrenoceptors. This classification was generally accepted and supported by the development of subtype-selective agonists and antagonists, and the therapeutic application of several of these pharmacological classes (Lands et al. 1967). But with the identification of selective agonists, and the expression of a recombinant receptor having similar characteristics, a third beta-adrenoceptor subtype, designated $\beta$3-adrenoceptor has been added to the list (Bylund et al. 1994). This $\beta$3-adrenoceptor was initially referred to as 'atypical $\beta$-adrenoceptor' (Esbenshade 1992, Liggett 1992) because of its insensitivity to the commonly used antagonists for $\beta$-adrenoceptors.

All of the $\beta$-adrenoceptors identified in pharmacological studies have been cloned, recombinant and expressed in a variety of tissue sources, including turkey, mouse, rat and human (Dixon et al. 1986, Yarden et al. 1986, Frielle et al. 1987, Emorine et al. 1989). The molecular pharmacology of $\beta$-adrenoceptors has been studied extensively, serving as one of the prototypes for the use of techniques such as site-directed mutagenesis to study the mode of interaction between the receptor and either agonists / antagonists or second-messenger regulatory proteins (Bylund et al. 1994), and studies revealed amino acid sequences with a high degree of homology between and within subfamilies.

All three $\beta$-adrenoceptor subtypes can be activated by endogenous catecholamines. However, in contrast to the alpha-adrenoceptors, E and NE do have differential affinity for the $\beta$-adrenoceptor subtypes. The potency orders are ISO>E=NE for $\beta$1-, ISO>E>>NE

for $\beta$2-, and ISO>NE>E for $\beta$3- adrenoceptors (Ruffolo 1994). They are linked to guanine nucleotide regulatory (stimulatory) proteins ($G_s$-proteins), which activate membrane-bound adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) inside the cell as a second messenger (Ruffolo 1994). The cAMP activates protein kinase A, which phosphorylates proteins leading to physiological responses. There is no evidence for subtype-related differences in receptor-cyclase interaction (Tate et al. 1991). However, there is evidence to suggest that in certain tissues, such as cardiac muscle, there could be a direct coupling between a $G_s$-protein and a voltage-sensitive calcium channel (Yatani et al. 1988).

The three $\beta$-adrenoceptor subtypes are closely related and may coexist within tissues (Galitzky et al.1993) and in the same cells (del-Monte et al. 1993). The location of $\beta$-adrenoceptors determines their functions, including cardiac effects, renal renin release and lipolysis for $\beta$1-, facilitation of NE release, vascular, bronchial and uterine smooth muscle relaxation, glycogenolysis in skeletal muscle and liver, and thermogenesis and protein turnover in skeletal muscle for $\beta$2-, cardiac effect, liver glycogenolysis, and lipolytic and thermogenic responses in adipose tissue for $\beta$3-adrenoceptors (Minneman et al. 1979a, Choo et al. 1992, Liggett 1992, Yanagisawa 1992).

#### 1.3.2.1 Beta-Adrenoceptors in Skeletal Muscles

Evidence supports the existence of all three $\beta$-adrenoceptor subtypes in skeletal muscles. Thorin et al. (1986) reported that the adrenoceptor mediating thermogenesis in muscles is the $\beta$1 subtype, and Rothwell et al. (1986) showed that $\beta$1-adrenoceptors had thermogenic

effects. However, this suggestion has not been supported by the work of Fagher et al. (1986, 1988) which showed a major thermogenic role for $\beta$2-subtype. Other reports suggested hypothermic effects in the cold for $\beta$2 receptor; stimulation of $\beta$2-adrenoceptors, by ip injection of agonists, produced hypothermic effects in the cold, whereas stimulation of $\beta$1-subtype had no direct effect, but antagonized the $\beta$2-elicited hypothermia when both subtypes were activated simultaneously (Carlisle and Stock 1993). The reasons for the different results are not yet known.

Rat *soleus* muscles were found to have both $\beta$1- and $\beta$2- adrenoceptors (Kim et al. 1992). However, Elfellah and Reid (1987) concluded that $\beta$-adrenoceptors in *gastrocnemius* and *soleus* muscles of pigs are predominantly, if not exclusively, of $\beta$2-subtype. This is supported by evidence that *soleus* muscles of cat (Minneman et al. 1979a), bovine *musculus trapezium* (Ijzerman et al. 1984) and *plantaris* muscles of rats (Kim et al. 1992) were found to have $\beta$2-subtype only. A functional role of $\beta$2-adrenoceptors in muscle protein accretion and thermogenesis also supports the presence of $\beta$2-adrenoceptors in skeletal muscle (MacRae et al. 1988, Astrup et al. 1989, Choo et al. 1992).

The presence of $\beta$3-adrenoceptors in skeletal muscle was suggested with evidence that BRL 28410 (a $\beta$3-agonist) stimulates oxygen consumption in the rat hind limb and also stimulates lactate formation and inhibits glycogen synthesis in rat *soleus* muscle (Arch and Kaumann 1993). This was supported by autoradiographic demonstration of $\beta$3-agonist binding in sections of rat *gastrocnemius*, *plantaris* and *soleus* muscles (Arch and Kaumann 1993).

### 1.3.2.2 Beta-Adrenoceptors in Heart

There has been a suggestion that heart contains all three $\beta$-adrenoceptors, with a relatively high proportion of $\beta$1-adrenoceptors. Hedberg et al. (1980) reported an apparent homogenous population of $\beta$1-adrenoceptors in the left ventricle of cat and guinea pig, whereas the atria of both species contained both $\beta$1- and $\beta$2- adrenoceptors in the ratio of approximately 3:1. These results are in agreement with physiological data reported by Carlsson et al. (1977) for cat heart, suggesting that both subtypes of adrenoceptors are responsible for the chronotropic control of the sino-atrial node. In contrast, data from O'Donnell and Wanstall (1979) on guinea pig heart suggest that only $\beta$1-adrenoceptors are involved in the chronotropic response, although $\beta$2-adrenoceptors are also present.

Minneman et al. (1979a) did not find $\beta$2-adrenoceptors in rat ventricle, which is in contrast to other findings which suggest small percentages of $\beta$2-adrenoceptors. These studies indicate presence of both $\beta$1- and $\beta$2-adrenoceptors in the ratio of 4:1 in the rat heart (Minneman et al. 1979b), 3:1 in the human heart (Bjornerheim et al. 1991b), and 3:2 in the rat heart (Rothwell et al. 1985). Robberecht et al. (1983) reported a much higher proportion of $\beta$2-adrenoceptors in the human auricles with 50 % $\beta$1- and 50 % $\beta$2-adrenoceptors. Bristow et al. (1986) reported the presence of $\beta$1- and $\beta$2- adrenoceptors in the ratio of 3:1 in the non-failing human ventricle and 3:2 in the failing human ventricles, and concluded that there is a selective downregulation of $\beta$1-adrenoceptors in the failing human heart, and as a result, the $\beta$2-adrenoceptor subpopulation is a relatively important

mediator of inotropic support (increased force of contraction through action on myocardial cells).

In the case of the presence of $\beta$3-adrenoceptors in heart, only one report (Yanagisawa 1992) supported a role for $\beta$3-adrenoceptors in cardiac functions.

#### 1.3.2.3 Beta-Adrenoceptors in Kidney

In mammalian kidney, catecholamines are involved in a number of functions, including regulation of renal blood flow, renin release, erythropoietin production and tubular function (Insel and Snavely 1981), indicating that $\beta$-adrenoceptors are present in the kidney. Gavendo et al. (1980) have reported that tubular cell membranes prepared from rat kidney contain $\beta$1-adrenoceptors, based on the rank order of potency of non-selective agonists in competing for [$^3$H]dihydroalprenolol binding sites. However, Snavely et al. (1982) indicated the presence of both $\beta$1- and $\beta$2-adrenoceptors in rat renal cortex in the ratio of 7:3. In another report by Snavely et al. (1985a) the ratio of $\beta$1- to $\beta$2-adrenoceptors in rat renal cortex was 3:2, which is different from a ratio of 3:1 also reported by the same authors (1985b). They concluded that the number of adrenoceptors and the ratio of $\beta$1- to $\beta$2- adrenoceptors depend on the level of circulating catecholamines in blood plasma and also on their differential regulation.

A number of reports support the hypothesis that the $\beta$-adrenoceptor regulating renin release is of the $\beta$1-subtype (Johns 1980, Kopp 1980, Nakane et al. 1980, Osborn et al. 1981). These receptors are probably located on the juxtaglomerular cells from which renin is released (Reids et al. 1978). The work of Gavendo et al. (1980) suggests that $\beta$1-

adrenoceptors may also be located on the tubules, but others disagree with this report because of the data suggesting alpha-adrenoceptor regulation of ions and water transport in the proximal tubule (Kim et al. 1980). Due to a variety of preparations used for radioligand binding assays, one cannot precisely localize $\beta$-adrenoceptors in the different kidney tissues.

#### 1.3.2.4 Beta-Adrenoceptors in Liver

Catecholamines regulate a wide variety of functions in the liver, including key steps in carbohydrate, lipid, and amino acid metabolism, and liver regeneration (Hadley 1984, Cruise et al. 1985). Whereas much descriptive information is available on the metabolic effects of catecholamines in liver, little published information exists on the characteristics of binding of $\beta$-adrenergic ligands to either homogenates or plasma membranes from this tissue. Ruffolo (1994) reported that $\beta$2- and $\beta$3- adrenoceptors stimulate glycogenolysis in liver. McDowell and Annison (1991) had earlier reported that glycogenolysis in liver is under the control of $\beta$2-adrenoceptors. Using radioligand binding assays, Kawai and Arinze (1983) showed that the potency order for the displacement of bound [$^3$H]dihydroalprenolol to purified rabbit liver, ISO>E>NE, which indicates the presence of $\beta$2-adrenoceptors, is similar to glycogenolytic effects of catecholamines. They concluded that the $\beta$-adrenoceptors that mediate glycogenolysis in liver are predominantly, if not exclusively, of $\beta$2-subtype.

Wolfe et al. (1976) had earlier characterized mammalian liver $\beta$-adrenoceptors using rat liver membrane and dihydroalprenolol. These studies, however, were not designed to

assess the proportions of receptor subtypes in this tissue, since dihydroalprenolol is a selective antagonist for $\beta$1- and $\beta$2- subtypes. However, Bendeck and Noguchi (1985), using radioligand binding studies, confirmed the presence of $\beta$2-adrenoceptors in liver. Recently, functional studies confirmed involvement of $\beta$2-adrenoceptors in liver glycogenolysis, and radioligand binding and molecular studies also confirmed the presence of $\beta$2-adrenoceptors in rat liver (Ishac et al. 1992).

### 1.3.3 Regulation of Beta-Adrenoceptors

A number of factors can elicit changes in the responsiveness of tissues to hormones. In many cases, this is due to a change in receptor number, but a change in receptor number does not always result in altered responses. There may be changes in maximal response, in sensitivity (concentration of a hormone required to produce half-maximal effect), or a combination of both of these factors (Brody 1994). Changes in hormone responsiveness may also be due to changes in receptor affinity or through modification of the signal transduction systems following hormone binding. The concentrations of hormone required for occupancy of the receptor and for the elicitation of a specific biological response often are similar, and significant changes in biological responses occur when receptor concentration or the binding affinity changes but hormone concentration remains constant. Otherwise, there is a marked dissociation of binding and the effect, and a maximum bioeffect occurs when only a small percentage of the receptors are occupied (Granner 1995), and the receptors not involved in the elicitation of the response are called

'spare receptors'. Therefore, the maximum binding capacity (Bmax) may not always indicate the amount of biological responsiveness.

Radioligand binding and molecular techniques have demonstrated alterations in the beta-adrenoceptor binding sites in a number of pharmacological, physiological, and pathological states. Bao (1990) found that the number of myocardial $\beta$-adrenoceptors increased while its affinity decreased progressively following endotoxin injection in rats. Density of $\beta$-adrenoceptors decreased in hypertensive rats (Watanabe et al. 1991), but increased in adrenalectomized rats (Kawai and Arinze 1983). In male rat adipocytes, testosterone stimulated catecholamine-induced lipolysis *in-vivo* by increasing the number of $\beta$-adrenoceptors as well as the activity of adenylate cyclase (Xu et al. 1991). Glucocorticoids have been shown to up-regulate $\beta$-adrenoceptors (Reynisdottir et al. 1993, Kiely et al. 1994). Insulin has a negative effect (Kang et al. 1993), growth hormone has a positive effect (Beauville et al. 1992), while thyroid hormones have both negative and positive effects (Liggett et al. 1989, Bjornerheim et al. 1991a) on the numbers of $\beta$-adrenoceptors and / or tissue responsiveness to catecholamines.

Other factors that affect the density of $\beta$-adrenoceptors include tissue location, and sex, age and the nutritional state of the animal. There is a marked regional variation in adrenoceptor number. In humans, abdominal fat cells have more $\beta$-adrenoceptors than gluteal fat cells (Arner et al. 1990). The number or subtype of adrenoceptors also depends on the type of tissue; in rats, a population of $\beta$-adrenoceptors is mainly $\beta 1$ in the heart and $\beta 2$ in the lung (Minneman et al. 1979b). Young male rats have a higher density of $\beta$-adrenoceptors in abdominal adipose tissue than female rats and older male rats (Studer

1987, Xu et al. 1991). The difference was attributed to the high level of testosterone in young male rats. Casteilla et al. (1994) determined that $\beta$1-adrenoceptor mRNA increased as the message for $\beta$3-adrenoceptor declined with age in bovine perirenal adipose tissue. $\beta$-adrenoceptor density is significantly reduced in ventricular membranes from animals fed saturated fat diets compared to those fed polysaturated fat diets (Nicolas et al. 1991).

The levels of adrenergic agonists and / or antagonists also have a significant influence on the density of $\beta$-adrenoceptors. The density of $\beta$-adrenoceptors in frog erythrocytes decreased with increased level of plasma catecholamine (Mukherjee et al. 1976), and the receptor number increased 2- to 5- fold in adrenalectomized rats (Wolfe et al. 1976). Repeated or continuous exposure of receptors to agonist leads to desensitization (Caron and Lefkowitz 1993, Lohse 1993, Marullo et al. 1995), involving three processes that occur in a temporal sequence; uncoupling of the receptor from the Gs-protein due to phosphorylation of the receptor leading to a decrease in receptor affinity; removal of the receptors from the plasma membrane (internalization) leading to a rapidly reversible loss of the surface receptors; with prolonged exposure, a loss in the total number of the receptors (down-regulation) that is only slowly reversible, requiring new synthesis of receptors. Phosphorylation of the $\beta$-adrenoceptors is catalyzed by protein kinase A (PKA) and $\beta$-adrenergic receptor kinase ($\beta$ARK) (Collins et al. 1992, Marullo et al. 1995). Degradation of receptor proteins in lysosomes and destabilization of receptor mRNA could also cause desensitization (Collins et al. 1992, Marullo et al. 1995). On the other hand, recycling of the sequestered receptors and activation of gene transcription by cAMP-response element binding protein (CREBP) could lead to resensitization / up-

regulation (Marullo et al. 1995). In addition, exposure of the receptors to an antagonist, inhibition of synthesis or release, or reduced level, of catecholamine in blood plasma can increase $\beta$-adrenoceptor processing and number (Williams et al. 1984, Brody 1994. This phenomenon is called 'up-regulation', and can result in hypersensitivity.

## 1.4 METABOLIC AND ENDOCRINE RESPONSES TO COLD

Ruminants adapt to chronic cold exposure by using behavioural and physiological mechanisms. The ability to increase feed intake is an important response for ruminants in a cold environment, and it provides the animals with ability to adapt to, and to minimize the impact of, a low effective ambient temperature (Sasaki and Weekes 1984, Christopherson 1994). Kennedy (1985) showed that sheep increased voluntary feed intake of chopped forages by 8 to 26 % during cold exposure. Increased feed intake in cold environments is also supported by other authors (Young 1983, Sano et al. 1995), and this increase serves to counter the effects of reduced digestibility, and to supply more nutrients for thermogenesis (Sasaki and Weekes 1984).

Since cold temperature has an effect on feed intake, it may also have an effect on feed conversion efficiency. Reductions in the feed efficiency were reported for pigs at 10 °C fed *ad libitum* (Fuller 1965) and for cold-adapted calves (Scott et al. 1993) compared to animals in warmer environments. These reductions could be attributed to reduced nutrient digestibility and use of nutrients for thermogenesis. That an increased proportion of nutrients was used as a substrate for thermogenesis rather than growth was suggested by Scott and Christopherson (1993) and Scott et al. (1993), who reported reduced average

daily gain and reduced protein synthesis in the cold-adapted calves fed the same dietary level as the warm-adapted controls.

The cold-induced thermogenesis is the increase in the heat production above thermoneutral metabolism, when an animal is exposed to temperature below its lower critical temperature (Sasaki and Weekes 1984, Christopherson 1994). McBride and Christopherson (1984) and Sano et al. (1995) reported that chronic cold exposure increased the heat production. Chronic exposure to a cold environment can also increase resting heat production which persists for 1 - 2 weeks after animals are returned to a thermoneutral temperature (Young et al. 1989). Young et al (1989) indicated that the chronic increase in the heat production in the cold environments may increase the capacity of the animals to tolerate severe cold stress. A ruminant's increased basal metabolism would minimize the impact of the low effective ambient temperature (Young 1983). Cold exposure increased whole-body oxygen consumption (Thompson et al. 1975), and oxygen consumption has been shown to decrease in domestic animals following the use of $\beta$-adrenergic antagonists (Christopherson and Brockman 1989), thus suggesting involvement of $\beta$-adrenoceptors in resting energy metabolism.

Cold exposure also has effects on rectal temperatures; sheep exposed to cold had lower rectal temperatures than those in the warm environment (Degen and Young 1980), and rectal temperatures were elevated in steers kept at 35 °C (Blaxter and Wainman 1961).

Acute cold exposure increases sympatho-adrenomedullary activity (Sasaki and Weekes 1984), and plasma E and NE concentrations are increased during chronic cold

exposure (Christopherson et al. 1978, Graham et al. 1981). This suggests that $\beta$-adrenoceptors could play a role in the metabolic responses to cold. The reduced level of insulin secretion in the cold allows enhanced mobilization of substrates in response to elevated sympatho-adrenomedullary activity (Sasaki and Weekes 1984). In contrast, Scott and Christopherson (1993) reported increased plasma insulin concentrations in cold-adapted heifers, and suggested that the increase was directed towards providing substrates for thermogenesis. For example, the increased plasma insulin could lead to increased glucose uptake by skeletal muscles. It may be that the relative balance among insulin, glucagon and catecholamines may be more important than absolute concentration in determining substrate mobilization. Cold exposure resulted in increased plasma T3, T4 and cortisol concentrations (Graham et al. 1981, Kennedy et al. 1985). The elevated thyroid hormone levels may increase the thermogenic capacity of skeletal muscle by influencing mitochondrial structure and membrane $Na^+$ / $K^+$-ATPase (Gregg and Milligan 1987). The permissive and direct effects of cortisol (Deavers and Musacchia 1979) may potentiate the responses to catecholamines in the cold, allowing an enhanced rate of glucose and fatty acid turnover to be maintained, and also allowing the animal to rapidly respond to any further demand for substrate mobilization, for example, any further increase in the intensity of cold exposure (Sasaki and Weekes 1984).

## 1.5 METABOLIC AND ENDOCRINE RESPONSES TO FEED RESTRICTION

The nutritional state of an animal also has significant influences on metabolic and endocrine responses. Animals metabolize substrates from the body reserves and / or diets

to meet up their daily energy demands. In the case of animals with unlimited access to feed, increased intake often occurs when energy requirements are increased (Sano et al. 1995). However, for animals with restricted feeding, increasing energy requirements for maintenance due to any reason becomes a problem, and usually restricts the productivity of the animals. Degen and Young (1980) determined that sheep fed *ad libitum* levels of feed intake gained live-weight more rapidly than restricted sheep. Feed restriction also has effects on metabolic rates and rectal temperatures of animals. Degen and Young (1980) reported that *ad libitum* fed sheep had a higher oxygen uptake and higher rectal temperatures than restricted sheep. Kelly et al. (1993) determined that ewes fed 2 x maintenance exhibited higher whole-body oxygen consumption than either maintenance-fed or fasted ewes. The metabolic responses to feeding are part of the classical heat increment of feeding and may reflect, in part, the work of digestion and the metabolic costs of metabolising absorbed nutrients. Christopherson and Brockman (1989) have shown that metabolism in the portal-drained viscera and liver of sheep accounted for 19 - 23 % and 18 - 34 % of total metabolic rate, respectively. Kelly et al. (1993) also suggested that visceral tissues contributed substantially to energy requirements for maintenance.

Although the average daily gain was reduced, moderate feed restriction led to improved feed efficiency in some studies with beef cattle (Hicks et al. 1990) and lambs (Murphy et al . 1994). Although there is no direct evidence for the mechanisms by which feed restriction improved feed efficiency in these cases, Murphy and Loerch (1994) suggested factors such as reduced size of metabolically active visceral organs leading to

lower maintenance energy expenditures, reduced physical activity, and increased diet digestibility.

The physiological mechanisms involved in the changes in the metabolic rate or heat production during feed restriction may also involve endocrine factors. Hadley (1984) reported that any decrease in blood glucose level leads to an acute enhancement of sympatho-adrenal activity with resulting elevation in plasma catecholamines. These catecholamines are stimulatory to hepatic glycogenolysis and adipose tissue lipolysis (Arch and Kaumann 1993), thus releasing additional substrates from the body reserves to support the energy demands during feed restriction. On the other hand, Christopherson and Brockman (1989) reported that propranolol, a $\beta$-blocker, reduced the metabolic response of the portal-drained viscera to feeding, suggesting an adrenergic component to the metabolic response to feeding in sheep. Feed restriction is generally associated with lower plasma insulin concentrations in ruminants (Lobley 1992), reflecting the lower energy and protein supply. Although feed restriction is associated with decreased thyroid activity (Murphy and Loerch 1994), Ellenberger et al. (1989) have observed that both T3 and T4 were unaltered in feed-restricted steers. Whereas Beaver et al. (1989) observed increased T3 levels in steers fed low energy intakes, Scott and Christopherson (1993) suggested that the alterations in the endocrine system during cold exposure of restricted-fed heifers appear to be directed toward providing substrates for use in thermogenesis. Therefore, a variety of factors contributes to the metabolic effects of feeding level in ruminants and the possibility of an adrenergic component requires further evaluation, particularly in relation to thermal environments.

## 1.6 HYPOTHESES

I have indicated that catecholamines play vital roles in modulating the energy requirements of meat and milk producing animals, and in their abilities to cope with environmental stresses encountered under normal production and livestock management systems. I also indicated that catecholamines, epinephrine and nor-epinephrine, act on adrenergic receptors to effect physiological functions, and that changes to these functions can be effected at the receptor level or any other level in the signal transduction pathways. These changes could be caused by changes in the internal or external environment.

With this background information, coupled with the fact that there is little information concerning the location, concentration and activity of ovine beta-adrenoceptors, a series of studies was designed to investigate the following hypotheses.

1) Tissues of lambs contain subtypes of $\beta$-adrenoceptors.
2) Different subtypes of $\beta$-adrenoceptors are present in different proportions in different tissues.
3) Cold exposure affects the densities and binding affinities of $\beta$-adrenoceptors.
4) The effects of cold exposure on the densities and binding affinities of $\beta$-adrenoceptors are modulated by plane of nutrition.
5) Cold exposure and feed restriction affect expression of genes for $\beta$-adrenoceptors as indicated by changes in the level of mRNA.
6) Metabolic and endocrine functions associated with changes in catecholamine responsiveness are also affected by cold exposure and feed restriction.

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## CHAPTER 2

## Metabolic and Endocrine Responses to Cold and Feed Restriction in Ruminants

### 2.1 INTRODUCTION

When challenged by cold and / or feed restriction, ruminants attempt to maintain internal stability using certain behavioural and physiological mechanisms. Studies relating to the impact of cold exposure and feed restriction on metabolic processes in ruminant livestock have increased our understanding of the physiological mechanisms underlying changes in animal production in cold environments and in regions or seasons of limited feed availability. In a cold environment, ruminants adapt to minimize the impact of the low effective ambient temperatures through increase in food intake (Young 1983), resulting in a reduced lower critical temperature. Metabolic heat production is also increased in the cold environments (Thompson et al. 1975, Graham et al. 1981, Schaefer et al. 1982, McBride and Christopherson 1984b, Sasaki and Weekes 1984, Young 1989). In contrast to the above, feed restriction leads to reduced whole-body oxygen consumption and heat production (Degen and Young 1980, Kelly et al. 1993).

The physiological mechanisms involved in the changes in resting metabolic rate in the cold and during feed restriction have not been fully established, but are likely the result of endocrine factors which induce changes in background thermogenic processes in tissues (Christopherson 1994). However, the specific thermogenic mechanisms that may be induced by the endocrine factors are uncertain, but thyroid status is known to alter respiration associated with the activity of the sodium pump in the plasma membrane of liver cells (Gregg and Milligan 1987). Cold exposure elevated plasma concentrations of

thyroxine (T4), triiodothyronine (T3) and cortisol (Kennedy et al. 1977, Fregly et al. 1979, Sasaki and Weekes 1984), and reduced plasma insulin concentration (Christopherson and Thompson 1983, Sano et al. 1995). Feed restriction is associated with reduced plasma insulin concentrations (Lobley 1992) and decreased, unaltered or increased T4 and T3 concentrations (Beaver et al. 1989, Ellenberger et al. 1989, Murphy and Loerch 1994).

However, little information is available concerning simultaneous metabolic and endocrine responses to cold and feed restriction in ruminants. If food intake is restricted, the insufficient nutrient availability combined with an increased maintenance cost due to cold exposure may further restrict the productivity of the animals. On the other hand, it is not known whether feed restriction in a cold environment can suppress the normal increase in resting metabolism resulting from the environment. The objective of the present study was to determine the effects of cold-adaptation and feed restriction on average daily body weight gain, heat production, rectal temperature and profiles of some metabolic hormones. Another objective of this experiment was to establish different physiological states for subsequent assessment of adrenergic receptor responses in sheep.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Animals and Their Management

Twenty four Suffolk-cross, six months old wether lambs were randomly assigned to one of the four treatment groups (WA, WR, CA and CR). Each lamb, housed in a separate pen (180 x 90 x 90 cm) in controlled environment rooms and exposed to either warm (W;

23 ± 2 °C) or cold (C; 0 ± 2 °C) temperatures, received experimental diets of alfalfa pellets (17 % crude protein, 9.08 MJ ME $Kg^{-1}$ DM, see Appendix 3 for feed composition) at an *ad libitum* (A) or a restricted (R) level. The restricted level in the warm environment was 1.35 x maintenance, but in the cold environment, the restricted animals received about 30 % more feeds than those in the warm environment. The restricted levels of feeding were designed to allow the growing lambs to gain weight, and were based on published information on heat production and total body insulation of shorn sheep (NRC 1975, Christopherson and Young 1981, McBride and Christopherson 1984 a, b). Water and cobalt-iodized salt were available *ad libitum*. All lambs were shorn to a fleece depth of approximately 1 cm, and re-shorn at 3 wk intervals to maintain a relatively constant level of insulation. The care and handling of the lambs conformed to the guidelines established by the Canadian Council on Animal care.

The lambs were initially adapted to treatments for 28 d before the experiment. The experiment was divided into three periods of five weeks each. In periods one and three the treatments described above were imposed. During period two, based on visual assessment of the animals, WR and CR groups were removed from the experiment and these animals were fed *ad libitum* in a thermoneutral environment to prevent major declines in body condition prior to period three.

### 2.2.2 Body Weight Gain, Feed Intake and Feed Efficiency

Fresh feed was provided daily at 0800 h. Daily feed intake was recorded every morning by weighing feed refusals for each animal, and body weight was recorded once a week.

The daily feed allotment for restricted animals was adjusted weekly according to body weights. The feed intake and body weight were used to calculate average daily gain (ADG) and feed efficiency.

### 2.2.3 Metabolic Rate and Rectal Temperature

The metabolic rate was measured in the animal's acclimation environment during the last week of each period. Estimation of heat production was based on the measurements of oxygen consumption ($V_{O2}$) over a 6 h period from 0900 to 1500 h, using an open circuit respiratory pattern analyzer (Young et al. 1975) connected to a respiratory hood (75 x 70 x 45 cm), through which air was drawn at a rate of 70 - 75 L $min^{-1}$. The air flow rates, measured by a flow meter (Rotometer, Fisher and Porter, Warmister, PA) were corrected for standard temperature and pressure. The respiratory hoods, supported by individual stancheons within the environment room allowed normal access of the lambs to feed and water, and standing or lying activities were not restricted. This open-circuit system was calibrated with nitrogen as a zero gas (Young et al. 1984), and the heat production was calculated from $V_{O2}$ using the equation of McLean (1972). The difference between the metabolizable energy intake and the heat production (metabolic rate) gave the energy balance of the animal (Bennett 1972).

Rectal temperatures were taken between 0800 and 0915 h at 2 d intervals using a thermistor probe and thermistor-telethermometer (Fisher Scientific Inc.). The relationship between the rectal temperatures and metabolic rates was also determined. At the end of period three animals were slaughtered for tissue collection. Feed was witheld from all

animals for 24 h prior to slaughter by captive-bolt stunning and exanguination. A blood sample was collected prior to slaughter. Tissue samples were collected as described in chapters three and four.

### 2.2.4 Hormone Radioimmunoassays

Blood samples (10 mL) were taken via the jugular catheter at 0800 h before measurements of oxygen consumption and 1 h before slaughter. The plasma samples were analyzed for insulin, total T4 and T3, and cortisol. The hormones were analyzed with a solid-phase [$^{125}$I]-radioimmunoassay kit with antibody-coated tubes and human serum calibrators (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California).

## 2.3 STATISTICAL ANALYSIS

The results were calculated as means ± SEM (N = 6) for each group of lambs, and for each data set. A two-way analysis of variance using the GLM procedures (2 X 2 factorial) of SAS program (SAS 1990) was done to assess the effects of temperature and feed intake and their interaction. The differences among means were compared using Duncan multiple range test. The sources of variations were temperature, feed intake and the interaction between them.

## 2.4 RESULTS

### 2.4.1 Body Weight Gain, Feed Intake and Feed Efficiency

Tables 2.1 - 2.3 present results for the three periods of the experiment (as indicated in the methods section, no results were recorded for WR and CR groups in the second period while they were being refed at an *ad libitum* level, see Appendix 1 for body weight values). In the cold environment, the average daily gain (ADG) of CA was about three times that of CR ($P < 0.001$). Similarly, in the warm environment, the ADG of WA was more than two times that of WR ($P < 0.001$). When the two environments were compared, the ADG for CR and WR were not different ($P > 0.05$), but the ADG for CA was less in the first period, and more in the second and third periods, than that of WA ($P < 0.05$). These differences are reflections of the differences in the average daily feed intake. The average daily feed intake of CA was greater than those of WA and CR ($P < 0.01$), and that of WA was greater than that of WR ($P < 0.001$). The average daily feed intake of CR was greater than that of WR ($P < 0.05$) in the first period only. Although differences existed in ADG and average daily feed intake, there were no significant differences between WA and WR, and between CA and CR ($P > 0.05$) in terms of the efficiency of use of feed for gain. However, feed efficiency was reduced ($P< 0.01$) by cold temperature exposure in the first period. The effect of interaction of temperature and feed intake on ADG and feed efficiency was not significant in either period one or three ($P > 0.05$).

### 2.4.2 Metabolic Rate and Rectal Temperature

The main effect of feed intake on metabolic rate (expressed as heat production) was significant ($P < 0.001$), while that of temperature was only significant in the first period ($P < 0.05$). The effect of interaction of temperature and feed intake was also significant ($P < 0.05$). In the cold environment, the metabolic rate of CA was about two times greater than that of CR ($P< 0.001$). And in the warm environment, the metabolic rate of WA was greater than that of WR ($P < 0.1$), but the difference was less compared to that of the cold environment. Although heat production tended to be elevated in the cold, it was only in the first period that heat production of CA was greater than that of WA ($P < 0.05$). But with feed restriction, there was no significant difference between WR and CR ($P > 0.05$) when the two environments were compared (Tables 2.1 - 2.3).

The energy balance, derived from metabolizable energy intake and metabolic rate, showed significant main effects and interaction of temperature and feed intake in the third period ($P < 0.05$). In the cold environment, the energy balance of CA was greater than that of CR ($P < 0.05$). And, in the warm environment, WA had a higher energy balance than WR ($P < 0.05$). There was no difference between WA and CA, or between WR and CR ($P > 0.05$) when the two environments were compared. WR animals were in negative energy balance in the first and third periods, while CR animals were in negative energy balance in the first period.

The energy balance was linearly related to metabolizable energy intake (Fig. 2.1) and the regression equations were:

$$EB = - 0.41 + 0.67\ ME \quad (r^2 = 0.82, \text{warm environment}),$$

$EB = -0.26 + 0.48\ ME$ ($r^2 = 0.40$, cold environment),

where EB is energy balance (MJ $Kg^{-0.75}$ $d^{-1}$) and ME is metabolizable energy intake (MJ $Kg^{-0.75}$ $d^{-1}$). The energy balance increased with an increase in metabolizable energy intake. From figure 2.1, the energy requirements for maintenance (X-intercepts) were 0.62 MJ $Kg^{-0.75}$ $d^{-1}$ and 0.56 MJ $Kg^{-0.75}$ $d^{-1}$ in the warm and cold environments, respectively. These values were not significantly different ($P > 0.05$). But when only the *ad libitum* groups were considered, the energy requirements for maintenance were 0.47 MJ $Kg^{-0.75}$ $d^{-1}$ and 0.66 MJ $Kg^{-0.75}$ $d^{-1}$ in the warm and cold environments, respectively.

Rectal temperature of CA was greater than that of CR ($P < 0.001$) in the cold environment, and the rectal temperature of WA was greater than that of WR ($P < 0.001$) in the warm environment. Compared with WA, the rectal temperature was reduced in the CA group ($P < 0.001$). Similarly, the rectal temperature of CR was less than that of WR ($P < 0.001$) when the two environments were compared. These patterns were maintained in the three periods, with significant main effects ($P < 0.001$), but the effect of interaction of temperature and feed intake was not significant ($P > 0.05$). The rectal temperature was linearly related to metabolic rate (Fig. 2.2) and the regression equations were:

$RT = 37.22 + 0.66\ MR$ ($r^2 = 0.26$, warm environment),

$RT = 37.38 + 0.36\ MR$ ($r^2 = 0.36$, cold environment),

where RT is rectal temperature (°C) and MR is metabolic rate (W $Kg^{-1}$). The rectal temperature increased as the metabolic rate increased.

### 2.4.3 Plasma Hormone Concentrations

Plasma insulin concentration in the CA group was greater than that of the CR group ($P < 0.001$) in the cold environment. The concentration of plasma insulin was also greater ($P < 0.05$) in the *ad libitum* fed group (WA) than in the restricted group (WR) in the warm environment. When both environments were compared, cold temperature exposure increased ($P < 0.05$) plasma insulin concentration of the *ad libitum* fed group (CA). Concentrations of plasma T3 followed similar patterns as insulin (Tables 2.1 - 2.3), but the difference between CA and WA was not significant ($P > 0.05$) in the third period. Plasma concentrations of T4 were similar between CA and CR, and between CA and WA, but there were significant differences ($P < 0.05$) between CR and WR in the third period, and between WA and WR in the first period. In the cold environment, plasma cortisol concentration of CR was greater than that of CA ($P < 0.01$). Similarly, plasma cortisol concentration of WR was greater than that of WA in the warm environment ($P < 0.05$). Cold temperature exposure increased plasma cortisol concentrations in both *ad libitum* and restricted fed groups, and these patterns were maintained throughout the three periods. The effect of interaction of temperature and feed intake on plasma concentration of hormones was significant for T3 and T4 in the first period and for insulin in the third period($P \leq 0.05$).

Table 2.4 shows plasma hormone concentrations 1 h before slaughter after witholding feed for 24 h. The absolute plasma hormone concentrations of all groups resemble those of restricted groups in the first and third periods. Concentrations of insulin, T3 and T4 for WA were higher ($P < 0.05$) than those of WR, and the concentration of cortisol was

higher ($P < 0.05$) in CA group than in WA group. The main effect of feed intake resulted in reduced plasma T3 and T4 in restricted groups, the main effect of temperature resulted in increased plasma cortisol, while the interaction of temperature and feed intake on plasma T3 was significant ($P \leq 0.05$).

### 2.4.4 Carcass Weight

The main effect of feed intake was significant ($P < 0.001$), while that of temperature and interaction of temperature and feed intake was not significant ($P > 0.05$). In the cold environment, CA had a higher carcass weight than CR ($P < 0.001$). Similarly, in the warm environment, WA had a higher carcass weight than WR ($P < 0.001$). But comparism of the two environments did not indicate any significant differences between the two *ad libitum* fed groups or between the two restricted-fed groups, $P > 0.05$ (Fig. 2.3).

## 2.5 DISCUSSIONS

### 2.5.1 Body Weight Gain, Feed Intake and Feed Efficiency

By design, the daily feed intakes of the restricted groups (WR and CR) were lower than those of the *ad libitum* fed groups (WA and CA), because animals in the WR and CR groups were maintained at restricted levels. The increased voluntary feed intake in the cold-exposed *ad libitum* fed group (Tables 2.1 - 2.3) is similar to findings reported by Young (1983), Kennedy (1985) and Sano et al. (1995). Egan (1977) reported that the increased voluntary feed consumption in the cold is facilitated by increased digesta passage rates and enhanced by improved protein supply to the intestine. This ability to

increase feed intake is an important response for ruminants in a cold environment, and it provides the animals with the ability to adapt to, and to minimize the impact of, the low effective ambient temperature in order to achieve reasonable levels of productivity (Sasaki and Weekes 1984, Christopherson 1994). The increased voluntary feed consumption serves to counter the effects of reduced digestibility (Kennedy 1985) and to supply more nutrients for thermogenesis in the cold.

Both feed restriction and cold temperature exposure had significant effects on the average daily gain (ADG) of the wether lambs. In both environments, feed restriction reduced the ADG of the wethers, but cold temperature exposure reduced in the first period, and increased in the second and third periods, the ADG of the *ad libitum* fed wethers. This could be due to adaptation to environment after a prolonged exposure to cold. These changes in ADG may be related to significant changes in the average daily feed intake, with increased feed intake leading to increased ADG, and *vice versa*. These results are similar to the reports that a low level of food was either insufficient to maintain the sheep, or resulted in a small retention of energy while a high level of food resulted in a considerable retention (Graham et al. 1959). Scott and Christopherson (1993) and Scott et al. (1993) reported reduced ADG and protein synthesis in the cold-adapted calves fed at the same level as their warm-adapted counterparts, and suggested that an increased proportion of the nutrients was used as a substrate for thermogenesis rather than growth. This may partly explain why the ADG of CA was less than that of WA in the first period, and the increased ADG of CA in the second and third periods

could partly be attributed to the large amount of feed consumed, possibly, an adaptation after a prolonged cold exposure.

There were no significant differences in efficiency of use of feed for gain between the *ad libitum* and restricted fed groups in the two environment despite the differences in the ADG and the average daily feed intake. Compared with the amounts of feed consumed, there was a low efficiency for the *ad libitum* fed groups and an improved efficiency for the restricted groups. This indicates that a larger proportion of feed consumed by the *ad libitum* fed groups was used to release energy for maintenance, rather than for growth. This result is similar to the works of Hicks et al. (1990) and Murphy et al. (1994) who reported that moderate feed restriction led to improved feed efficiency in beef cattle and lambs, respectively. Although no direct evidence has been reported for the mechanisms by which feed efficiency is improved with moderate feed restriction, Murphy and Loerch (1994) suggested factors such as reduced size of metabolically active organs leading to lower maintenance energy expenditures, reduced physical activity, and increased diet digestibility. Cold temperature exposure reduced feed efficiency in both *ad libitum* and restricted groups during the first period, and this may relate to the reduced ADG observed. Similar reductions in efficiency were reported for pigs at 10 °C fed *ad libitum* (Fuller 1965) and cold-adapted calves (Scott et al. 1993). This could be attributed to reduced nutrient digestibility (Kennedy 1985) and use of nutrients for thermogenesis (Scott et al. 1993).

The absolute values of ADG and feed efficiency obtained in this study agree with some of the previous findings. Sano et al. (1995) reported ADG values of 0.03 $Kg.d^{-1}$ and

-0.14 $Kg.d^{-1}$, and feed efficiency values of 0.03 $Kg.Kg^{-1}$ and -0.12 $Kg.Kg^{-1}$ for warm and cold exposed one year old rams, respectively. A report by McBride and Christopherson (1984a) indicated ADG values of 0.15 - 0.24 $Kg.d^{-1}$ and 0.14 - 0.20 $Kg.d^{-1}$, and feed efficiency values of 0.35 $Kg.Kg^{-1}$ and 0.37 $Kg.Kg^{-1}$ for warm and cold exposed 10 wk old lambs, respectively. The present study indicated ADG values of 0.10 - 0.32 $Kg.d^{-1}$ and 0.06 - 0.34 $Kg.d^{-1}$, and feed efficiency values of 0.07 - 0.15 $Kg.Kg^{-1}$ and 0.07 - 0.10 $Kg.Kg^{-1}$ for the warm and cold exposed wethers, respectively. The observed differences could be due to the different types of diets used and the ages of the animals. Different types of diets have different digestibilities and metabolizable energy contents, and younger animals grow faster than the older ones.

### 2.5.2 Metabolic Rate and Rectal Temperature

In both cold and warm environments, feed restriction reduced heat production in lambs, and these results agree with previous findings. Kelly et al. (1993) have demonstrated that ewes fed 2 x maintenance exhibited higher whole-body oxygen consumption than either maintenance or fasted ewes. And Degen and Young (1980) reported that *ad libitum* sheep had a higher oxygen uptake than restricted sheep in the warm environment. But the report by Degen and Young (1980) did not indicate any differences between *ad libitum* and restricted sheep in the cold environment, as indicated in the present study. The reduced heat production in feed restricted groups could be a way of reducing the maintenance energy requirement. This adaptation to low energy intakes is likely to be important to animal survival during periods of scarce feed supplies.

The interrelationships between the feed intake and heat production of these shorn sheep and the thermal environment are similar to those reported by Blaxter (1967). Metabolic rate of cold-adapted *ad libitum* fed lambs was higher than that of lambs in the warm environment. But this difference was only significant in the first period. Similar results were reported for shorn ewes (McBride and Christopherson 1984a), and cold-adapted calves (Webster et al. 1978, Scott et al. 1993). It is probable that metabolizable energy was diverted away from tissue growth toward heat production in the cold-exposed lambs. Young et al. (1989) indicated that the chronic increases in heat production in a cold environment may increase the capacity of the animals to tolerate severe cold stress. This cold-induced thermogenesis is a homeostatic response that permits animals to increase heat production using substrates metabolized from body tissues or arising from dietary metabolizable energy. For animals with unlimited access to feed, increased intake compensates for the increased energy requirement, but for those with restricted feeding, requirements for thermogenesis reduce metabolizable energy available for protein deposition, since net protein deposition is positively related to the supply of metabolizable energy (Reeds and Fuller 1983).

Reports by Graham et al. (1959) indicated that when sheep are shorn the heat production increased at temperatures below 18 °C. However, this study shows that the sheep in CR group had similar metabolic rates as those in WR group. The sheep in the CR group were expected to have higher levels of heat production to maintain their body temperatures. Bennett (1972) reported that summit metabolism was 26 - 28 % lower when the sheep were lying than when they were standing. During the present study, lying

and standing activities were not restricted, and the sheep in CR group may have spent more time lying than standing. This could partly explain the low metabolic rates in this group. Unfortunately, times spent standing versus lying were not recorded.

The sheep in WR group were in negative energy balance, although they had similar ADG as those in CR group. These inconsistent results may be related to an unusual relatively high and low metabolic rates exhibited by the sheep in WR and CR groups, respectively. These results were reflected in the findings that the mean values of energy requirements for sheep in the cold and warm environments were similar when all the sheep in each environment were considered. This could be attributed to an effect of feed restriction, since the energy requirement in the cold environment was 40 % higher than that of the warm environment when only the sheep in the *ad libitum* fed groups were considered. The negative energy balance for the restricted groups in the first period did not reflect in the weight gains recorded for these groups. It might be that the animals were only in negative energy balance at the times of measurements.

Both cold temperature exposure and feed restriction reduced rectal temperature of the lambs. This reduction was significant and is similar to previous reports. Blaxter and Wainman (1961) reported elevated rectal temperatures for steers kept at 35 °C, and Degen and Young (1980) reported that *ad libitum* fed sheep had higher rectal temperatures than the restricted ones, in both cold and warm environments. The reduced rectal temperature could be an adaptation to a low level of energy intake, thus reducing the amount of energy needed to maintain the body temperature. A higher body temperature in the cold could increase the temperature difference between the body and the environment, increasing the

gradient for heat loss. Therefore, there would be an advantage for the animal to maintain a low body temperature in a cold environment.

The approximately linear relation between rectal temperatures and metabolic rates found in sheep is in accordance with the study by Bennett (1972). The rectal temperature increased as the metabolic rate increased. The fact that lying reduced both metabolic rate and rectal temperature (Bennett 1972) could partly explain the low metabolic rates and rectal temperatures exhibited by sheep in the CR group. Extrapolation of the curve (Fig. 2) to 'zero metabolism' gave 37 °C, approximately, as the 'temperature of zero metabolism' in the cold and warm environments. This value is higher than 25 °C reported for sheep by Bennett (1972). The difference could be due to breed or the wide range of rectal temperatures (30 - 40 °C) as opposed to a range of 37 - 41 °C for the present study. In addition, Bennett (1972) reported that there was no consistent relation between metabolism and rectal temperatures above 37 °C.

### 2.5.3 Plasma Hormone Concentrations

Feed restriction is generally associated with lower plasma insulin concentrations in ruminants (Brockman and Laarveld 1986, McCann and Hansel 1986, Lobley 1992), reflecting the lower energy and protein supply. In the present study, feed restriction resulted in reduced plasma insulin concentrations in the cold and warm environments. The reduced plasma level of insulin allows enhanced mobilization of substrates in response to elevated sympatho-adrenomedullary activity (Sasaki and Weekes 1984). Cold temperature exposure increased plasma insulin concentrations of the *ad libitum* fed group.

This result agrees with the reports for cold-adapted heifers (Scott and Christopherson 1993), but it is in contrast to reduced plasma insulin concentration in the cold reported for sheep (Sano et al. 1995) and bulls (Christopherson and Thompson 1983). In addition, Sasaki et al. (1982) reported unaltered basal insulin levels for sheep in the cold. The reason for these inconsistent reports is not known, but it could be related to the physiology of the animals studied.

The present study shows that feed restriction reduced the plasma T3 concentrations, but did not have any effects on plasma T4 concentrations in lambs. Although feed restriction is associated with decreased thyroid activity (Blake et al. 1991, Murphy and Loerch 1994), Ellenberger et al. (1989) reported that both plasma T3 and T4 were unaltered in feed-restricted steers, whereas Beaver et al. (1989) observed increased T3 levels in steers fed low energy intakes. The reduced thyroid secretion is an indication of reduced energy requirements for maintenance resulting in a greater proportion of the total energy consumed being used for growth rather than would normally be expected, and hence, improved feed efficiency (Murphy and Loerch 1994). Cold exposure resulted in increased plasma T3 and T4 levels in lambs in the present study, although some of the increases were not significant (Tables 2.1 - 2.3). These results are similar to previous reports for sheep (Kennedy et al. 1977 and 1985) and for heifers (Scott and Christopherson 1993). The elevated thyroid hormone levels may increase the thermogenic capacity of the skeletal muscles by influencing mitochondrial structure and membrane $Na^+ / K^+$ - ATPase (Gregg and Milligan 1987).

Cold temperature exposure and feed restriction increased plasma cortisol concentrations in lambs (Tables 2.1 - 2.3). Similar results were reported by Faulkner et al. (1980), Graham et al. (1981) and Thompson et al. (1982). Cold exposure probably increased the activity of the hypothalamo-pituitary-adrenocorticoid system resulting in increased plasma concentrations of cortisol. The permissive and direct effects of cortisol (Deavers and Musacchia 1979) will potentiate the responses to catecholamines in the cold, allowing an enhanced rate of glucose and fatty acid turn-over to be maintained during continued cold exposure, supplying energy for thermogenesis in skeletal muscles, and also allowing the animals to rapidly respond to further demands for substrate mobilization.

When all the lambs were fasted for 24 h, the plasma hormone concentrations of all groups were similar to those observed for the restricted groups in the three periods of experimentation (Table 2.4). The plasma hormone concentrations of the *ad libitum* fed groups were similar to the levels observed for the restricted groups during the three periods. The withdrawal of feed and water affected the *ad libitum* groups more than the restricted groups. This clearly shows the importance of nutritional factors in regulation of endocrine responses.

### 2.5.4 Carcass Weight

The treatment groups developed the differences in carcass weight that have been observed in other animals subjected to similar treatments. Williams et al. (1984) observed reduced body weight with feed restriction in rats. In the cold and warm environments, feed

restriction significantly reduced the carcass weights of the lambs. The reduction in carcass weight by feed restriction was expected, and is related to the reduced ADG due to feed restriction. Cold temperature exposure did not have any effects on carcass weight, indicating that the influence of feed restriction was more important than that of cold exposure.

## 2.6 CONCLUSION

It is concluded that ruminant animals undergo alterations in physiological responses due to cold exposure and / or feed restriction. In this study, cold exposure reduced rectal temperatures of lambs, but increased metabolic rates and plasma levels of insulin, T3 and cortisol. Feed restriction increased plasma cortisol level, but reduced ADG, metabolic rates, energy balance, rectal temperatures and plasma insulin and T3 levels. The alterations in endocrine responses may be directed toward providing substrates for use in thermogenesis or release of energy for maintenance. The reduced metabolic rates and the improved efficiency of use of feed in feed restricted groups may be adaptations to low energy intakes, and are likely to be important to ruminant animals' survival during periods of scarce feed supplies. However, insufficient nutrients combined with increased maintenance cost in the cold ultimately restricts the productivity of ruminant animals. This is because the lambs in the CR group had the lowest carcass weights.

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**Table 2.1** Effects of temperature and feed intake on metabolic and endocrine profiles[Z] of lambs during the first period

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| ADG[Y] (Kg) | 0.21a | 0.14b | 0.27c | 0.08d | 0.02 | 0.22 |
| Feed (Kg $d^{-1}$) | 1.46b | 1.77a | 2.39c | 0.85d | 0.04 | 0.004 |
| FE[X] (Kg $Kg^{-1}$) | 0.14a | 0.08b | 0.11 | 0.10 | 0.01 | 0.94 |
| MR[W] | 0.66b | 0.78a | 0.85c | 0.59d | 0.05 | 0.05 |
| En-bal[V] | 0.10 | 0.12 | 0.34c | -0.11d | 0.05 | 0.23 |
| RT[U] (°C) | 39.4a | 38.6b | 39.5c | 38.5d | 0.06 | 0.13 |
| Insulin (μIU $mL^{-1}$) | 9.9 | 13.1 | 17.4c | 5.6d | 1.58 | 0.09 |
| T3[T] (ng $dL^{-1}$) | 104.9b | 157.8a | 178.1c | 84.8d | 12.26 | 0.03 |
| T4[S] (μg $dL^{-1}$) | 5.3 | 5.4 | 5.6 | 5.1 | 0.34 | 0.03 |
| Cortisol (μg $dL^{-1}$) | 1.66b | 3.71a | 1.76d | 3.62c | 0.30 | 0.22 |

[Z]Values are means, N = 6.
[Y]Average daily gain.
[X]Feed Efficiency.
[W]Metabolic rate in MJ $Kg^{-0.75}$ $d^{-1}$.
[V]Energy balance in MJ $Kg^{-0.75}$ $d^{-1}$.
[U]Rectal temperature.
[T]Triiodothyronine.
[S]Thyroxine.
a, b, or c, d indicate main effects of temperature or feed intake that are different ($P < 0.05$).
T*F indicate probabilities for interaction of temperature and feed intake

**Table 2.2** Effects of temperature and feed intake on metabolic and endocrine profiles[z] during the second period

| | Temperature | | SEM | T*F |
|---|---|---|---|---|
| | Warm | Cold | (Pooled) | Probability |
| ADG[y] (Kg) | 0.22b | 0.34a | 0.04 | 0.05 |
| Feed (Kg $d^{-1}$) | 2.88b | 3.37a | 0.12 | 0.01 |
| FE[x] (Kg $Kg^{-1}$) | 0.08 | 0.10 | 0.01 | 0.14 |
| MR[w] | 0.88 | 0.98 | 0.09 | 0.45 |
| En-bal[v] | 0.37 | 0.47 | 0.10 | 0.48 |
| RT[u] (°C) | 39.80a | 39.20b | 0.06 | 0.0001 |
| Insulin (μIU $mL^{-1}$) | 13.08b | 24.65a | 2.11 | 0.003 |
| T3[t] (ng $dL^{-1}$) | 181.74b | 244.20a | 10.67 | 0.002 |
| T4[s] (μg $dL^{-1}$) | 7.63 | 8.42 | 0.67 | 0.42 |
| Cortisol (μg $dL^{-1}$) | 0.78b | 1.69a | 0.45 | 0.18 |

[z]Values are means, N = 6.
[y]Average daily gain.
[x]Feed Efficiency.
[w]Metabolic rate in MJ $Kg^{-0.75}$ $d^{-1}$.
[v]Energy balance in MJ $Kg^{-0.75}$ $d^{-1}$
[u]Rectal temperature.
[t]Triiodothyronine.
[s]Thyroxine.
a, b indicate main effects of temperature that are different ($P < 0.05$).

**Table 2.3** Effects of temperature and feed intake on metabolic and endocrine profiles[z] of lambs during the third period

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| ADG[y] (Kg) | 0.16 | 0.19 | 0.24c | 0.11d | 0.02 | 0.11 |
| Feed (Kg $d^{-1}$) | 2.31b | 2.60a | 3.38c | 1.53d | 0.08 | 0.17 |
| FE[x] (Kg $Kg^{-1}$) | 0.07 | 0.07 | 0.07 | 0.07 | 0.01 | 0.32 |
| MR[w] | 0.76 | 0.78 | 0.92c | 0.62d | 0.04 | 0.03 |
| En-bal[v] | 0.17b | 0.32a | 0.37c | 0.15d | 0.06 | 0.049 |
| RT[u] (°C) | 39.2a | 38.6b | 39.6c | 38.3d | 0.08 | 0.36 |
| Insulin (μIU $mL^{-1}$) | 12.3b | 15.6a | 18.5c | 9.4d | 1.19 | 0.05 |
| T3[t] (ng $dL^{-1}$) | 137.5 | 152.6 | 178.6c | 111.5d | 8.86 | 0.34 |
| T4[s] (μg $dL^{-1}$) | 7.9b | 9.3a | 9.0 | 8.2 | 0.41 | 0.30 |
| Cortisol (μg $dL^{-1}$) | 1.17b | 2.51a | 1.22d | 2.46c | 2.01 | 0.60 |

[z]Values are means, N = 6.
[y]Average daily gain.
[x]Feed Efficiency.
[w]Metabolic rate in MJ $Kg^{-0.75}$ $d^{-1}$.
[v]Energy balance in MJ $Kg^{-0.75}$ $d^{-1}$
[u]Rectal temperature.
[t]Triiodothyronine.
[s]Thyroxine.
a, b, or c, d indicate main effects of temperature or feed intake that are different ($P < 0.05$).
T*F indicate probabilities for interaction of temperature and feed intake

**Table 2.4** Effects of temperature and feed intake on endocrine profiles[z] of lambs just before slaughter[y]

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Insulin ($\mu IU\ mL^{-1}$) | 6.28 | 5.66 | 6.80 | 5.14 | 0.70 | 0.13 |
| T3[x] ($ng\ dL^{-1}$) | 108.8 | 92.7 | 112.9c | 88.6d | 8.62 | 0.05 |
| T4[w] ($\mu g\ dL^{-1}$) | 5.67 | 5.56 | 6.16c | 5.07d | 0.30 | 0.56 |
| Cortisol ($\mu g\ dL^{-1}$) | 1.82b | 3.16a | 2.85 | 2.14 | 0.37 | 0.45 |

[z]Values are means, N = 6.
[y]All the wethers were fasted 24 h before slaughter.
[x]Triiodothyronine.
[w]Thyroxine.
a, b, or c, d indicate main effects of temperature or feed intake that are different ($P < 0.05$).
T*F indicate probabilities for interaction of temperature and feed intake

**Figure 2.1**. The relationship between energy balance of sheep and metabolizable energy intake in the third period. Regression lines were derived from least squares. Each point represents an individual value (N = 12).

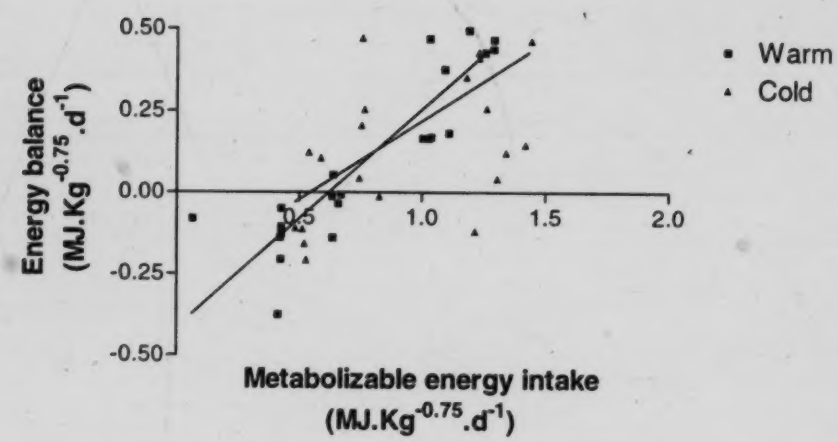


**Figure 2.2** The relationship between rectal temperatures and metabolic rates of sheep in the third period. Regression lines were derived from least squares. Each point represents an individual value (N = 12).

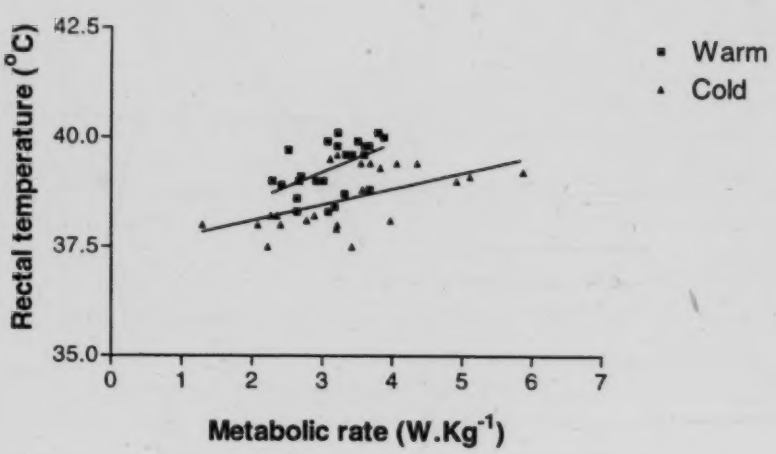
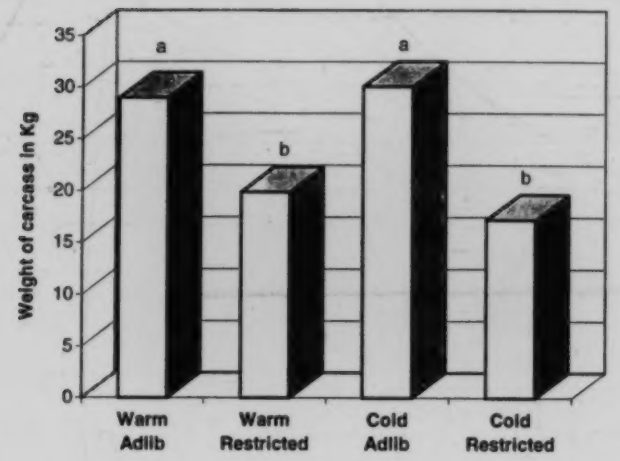


**Fig. 2.3** Weights of the carcasses (values are means, N = 6). Different superscripts indicate significant differences ($P < 0.001$).




The table below indicates the main effects of temperature and feed intake and their interaction on carcass weights. Only the main effect of feed intake was significant. ($P < 0.05$).

| Temperature | | Feed Intake | | Pooled | T*F |
|---|---|---|---|---|---|
| Warm | Cold | Adlib | Restricted | SEM | |
| 24.4 | 23.7 | 29.5c | 18.6d | 0.86 | 0.14 |

c, d indicate significant difference for feed intake.

## CHAPTER 3

# Beta-Adrenergic Receptors in Skeletal Muscles of Ruminants: Effects of Temperature and Feed Intake

## 3.1 INTRODUCTION

Tissue adrenergic receptors (adrenoceptors) mediate the numerous effects of endogenous catecholamines (epinephrine and norepinephrine) produced in the central nervous system, in peripheral sympathetic nerves, and / or in the adrenal medulla (Christopherson et al. 1995; Meyer et al. 1995). These adrenergic effects have been associated with increased milk and lean meat production, increased adaptability of animals to their environments, and reduced cost of animal production (Hanrahan 1987; Meyer et al. 1995).

Two major classes of adrenoceptors (alpha, $\alpha$ and beta, $\beta$) were originally described by Ahlquist (1948) on the basis of their differential responses to a variety of natural and synthetic agonists and antagonists. These main classes have been further divided into several subtypes; $\alpha$1 (a, b, c), $\alpha$2 (a, b, c, d), $\beta$1, $\beta$2 and $\beta$3 adrenoceptors (Arch and Kaumann 1993; Ruffolo et al. 1994). The $\beta$-adrenoceptors are linked to guanine nucleotide (stimulatory) protein, which activates adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) inside the cell as a second messenger. The cAMP activates protein kinase A, which phosphorylates proteins, leading to different physiological responses (Ruffolo et al. 1994).

Responses to adrenergic agents have been shown to be modified by environmental and nutritional conditions. Cold increases activities of the sympatho-adreno-medullary

system, leading to increased release of endogenous adrenergic agonists (Sasaki and Weekes 1984). In rats, fasting suppresses and overfeeding stimulates the sympathetic nervous system (Shetty and Kurpad 1990). Changes in release of endogenous catecholamines are expected to alter activation of adrenergically-mediated cellular events. For example, Graham and Christopherson (1981) reported that the thermogenic response to epinephrine was enhanced in cold-acclimated sheep. Modulation of adrenergic responses by tissues can occur at the level of the receptors, or at other levels in the signal transduction pathway. It is not clear whether altered responses to catecholamines occur at the receptor level as a result of different adrenoceptor densities, binding affinities or both.

Radioligand binding studies have demonstrated alterations in the $\beta$-adrenoceptor densities in a number of pathological and physiological states (Kawai and Arinze 1983, Bao 1990, Watanabe et al. 1991). However, there is no report on the influence of cold exposure or feeding level on expression of $\beta$-adrenoceptors in animals. The objectives of this study were to specifically investigate the co-existence and the relative densities of $\beta$1 and $\beta$2 adrenoceptors in skeletal muscles of lambs, and to determine whether the concentration and binding affinities of these receptors are affected by environmental temperature and plane of nutrition.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Animals and Management

Animals and treatments are the same as described in chapter 2. Twenty-four Suffolk-cross, six-month-old wether lambs were randomly divided into four groups (WA, WR,

CA and CR). The lambs, housed in individual pens (180 x 90 x 90 cm), were exposed to either warm (W; 23 ± 2 °C) or cold (C; 0 ± 2 °C) temperatures. The lambs received experimental diets of alfalfa pellets (17 % crude protein, 9.08 MJ ME.Kg$^{-1}$ DM; see Appendix 3 for feed composition) either *ad libitum* (A) or at a restricted level (R), on a daily basis. Based on published information on heat production and total body insulation of shorn sheep (NRC 1975, Christopherson and Young 1981, McBride and Christopherson 1984 a, b), the feed restriction level in the warm environment was 1.35 x maintenance and that of the cold environment was 30 % above the level in the warm environment. Water and cobalt-iodized salt were available *ad libitum*.

The lambs were shorn to a fleece depth of approximately 1cm. To maintain a relatively constant level of insulation, the wethers were re-shorn at 3-week intervals. Pens were regularly cleaned and bedded as required, and the lambs were regularly monitored for evidence of thermal stress. The care and handling of the wethers used in this study conformed to the guidelines established by the Canadian Council on Animal Care. The lambs were initially adapted to treatments for 28 d before the experiment. The experiment was divided into three periods of five weeks each. In periods one and three, the treatments described above were imposed. During period two, WR and CR groups were removed from the experiment and these animals were fed *ad libitum* in a thermoneutral environment to prevent major declines in body condition prior to period three.At the end of the third period, the wethers were slaughtered using captive bolt stunning and exsanguination, and *biceps femoris, semitendinosus* and *gastrocnemius* muscles were quickly harvested. Muscle subsamples were wrapped in aluminium foil and frozen in

liquid nitrogen within 15 min, prior to storage at -72 °C. All animals were fasted for 24 hr before slaughtering to reduce gut fill and facilitate the evisceration and tissue sampling process.

### 3.2.2 Membrane Preparation

Membrane preparation was performed according to methods of Ohlendieck et al. (1991). One hundred g of the frozen skeletal muscle was cut into pieces and homogenized three times (30 seconds, with 2 min intervals) in 700 mL of buffer A (see below) using a Waring blender. The homogenate was centrifuged using a JA-10 rotor at 14,000 x g for 15 min and filtered through 6 layers of cheese cloth. The pellet was homogenized again in 500 mL of buffer A, centrifuged and filtered. The combined supernatant was centrifuged using a JA-14 rotor at 30,000 x g for 30 min and filtered. Solid KCl was added to the filtrate to a concentration of 0.6 M. The treated filtrate was centrifuged with a 60 Ti-rotor for 35 min at 142,000 x g. All centrifugation was done at a temperature of 4 °C, and all glasswares were placed on ice. The pellet from each tube was resuspended in 2 mL of buffer B (see below), and frozen in 1 mL aliquots at -72 °C. These aliquots were used for determination of protein content and for radioligand binding assays. The protein concentration of the membrane extracts, in mg $mL^{-1}$, was determined by Bradford's test (Bradford 1976), using bovine serum albumin as a standard.

**Buffer A:** 20 mM tetrasodium pyrophosphate, 20 mM sodium phosphate, 1 mM magnesium chloride, 0.303 M sucrose, 0.5 mM ethylenediaminetetraacetic acid (EDTA),

76.8 nM aprotinin, 1.1 μM leupeptin, 0.7 μM pepstatin A, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.0.

**Buffer B:** 0.303 M sucrose, 20 mM tris-maleate, 0.6 M KCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0.

### 3.2.3 Radioligand Binding Assay

Binding of tritiated dihydroalprenolol ([$^3$H]DHA, specific activity 57.0 Ci mmol$^{-1}$, New England Nuclear) to membrane extracts was performed as described previously (Mersmann and McNeel 1992). Briefly, 25 μL of different concentrations of [$^3$H]DHA were added to a tube containing 25 μL of buffer B. One hundred μL of 1 mg.mL$^{-1}$ membrane was added to each tube and incubated for 30 min at 25 °C (total volume, 150 μL). The final concentrations of [$^3$H]DHA in the mixtures were between 0.08 and 20 nM. Five mL of ice-cold buffer B was added to each tube at the end of the incubation. Filtration was done using Whatman GF/B glass fibre (pre-soaked in 0.5 % polyethylenimine to reduce non-specific binding) mounted on a suction apparatus. The filters were washed twice with 5 mL of ice-cold buffer B. The filter-bound radioactivity, representing the total binding, was measured by liquid scintillation spectrometry.

Non-specific binding was estimated at each concentration of [$^3$H]DHA by using 25 μL of $10^{-5}$ M (±)-propranolol in place of buffer B. The difference between the total and non-specific bindings gave the specific binding. Since DHA is a selective antagonist for both $\beta$1 and $\beta$2 adrenoceptors, the specific binding indicates binding of [$^3$H]DHA to both receptor subtypes. A third binding experiment was done for each concentration of

[$^3$H]DHA in the presence of 5 μM terbutaline (Bylund et al. 1994), with an assumption that the terbutaline binds to all $\beta$2-adrenoceptors, and the estimate gave the binding to $\beta$1-adrenoceptors. Binding to $\beta$2- adrenoceptors was then estimated by calculation. Each experiment was performed in duplicate.

### 3.3 DATA ANALYSIS

The binding data were subjected to curve fitting procedures, using the non-linear regression analysis of Graphpad Prism (Graphpad 1995), to obtain density (maximum binding capacity, Bmax) of $\beta$1 and $\beta$2 adrenoceptors, and binding affinity (Kd) of the receptors. The results were analysed as a 2 X 2 factorial using GLM procedures of SAS (SAS 1990). The model included a two-way analysis of variance and a Duncan multiple range test. The differences among means were compared using the Duncan multiple range test. The sources of variations were temperature, feed intake and the interaction between them.

### 3.4 RESULTS

The specific binding was saturated for DHA equilibrium binding (Fig. 3.1), and all the Scatchard plots (plots of ratio of specific binding to free ligand concentration against specific binding) for specific binding were linear and fit with a correlation coefficient > 0.9. The equilibrium binding data could not be fitted to a two-site model, and there was no evidence for curvilinear Scatchard plots that might imply negative co-operativity or the presence of multiple binding sites.

For the *biceps femoris* muscle, only the main effect of feed intake on density of $\beta$1-adrenoceptors was significant ($P < 0.001$). The main effect of temperature approached a 5 % significant level ($P = 0.08$), and the interaction of temperature and feed intake was not significant ($P > 0.05$). The receptor density (Bmax) for $\beta$1-adrenoceptors was highest in CR group. In the cold environment, the Bmax for CR was significantly higher than that of CA ($P < 0.05$). And, in the warm environment, the Bmax for WR was higher than that of WA ($P < 0.05$). In both warm and cold environments (WA:WR; CA:CR), feed restriction increased ($P < 0.05$) receptor density (Fig. 3.2). The temperature effect was only significant ($P < 0.05$) with feed restriction, where cold temperature caused an increase in Bmax.

Figure 3.3 shows the Bmax of $\beta$1-adrenoceptors for *gastrocnemius* muscle. The Bmax value was highest in the CR group. In both warm and cold environments, the Bmax increased with feed restriction, but the difference was only significant in the cold ($P < 0.05$). The Bmax values for WA and CA were similar, and those of WR and CR were also similar ($P > 0.05$). Similar to results obtained for *biceps femoris*, the main effect of feed intake on the density of $\beta$1-adrenoceptors in *gastrocnemius* muscle was significant ($P < 0.01$) There was no indication of an effect of temperature, and the effect of interaction of temperature and feed intake was also not significant ($P > 0.05$). Similar results were obtained for *semitendinosus* muscle, where the Bmax value was also highest in the CR group (Fig. 3.4). In *semitendinosus*, the Bmax value for CR was higher than that of CA in the cold environment ($P < 0.05$). But in the warm environment, the Bmax value for WR was higher than, but not significantly different from that of WA ($P > 0.05$).

When both environments were compared, the Bmax values for WA and CA, and for WR and CR, were similar ($P > 0.05$). Only the main effect of feed intake was significant ($P = 0.001$).

Table 3.1 shows the effects of temperature and feed intake on the densities of $\beta$2-adrenoceptors in the three skeletal muscles of lambs. The main effects of temperature and feed intake and their interaction were not significant ($P \geq 0.05$). However, for *gastrocnemius* muscle, the main effect of feed intake on density of $\beta$2-adrenoceptors approached the 5 % significant level ($P = 0.08$).

Table 3.2 shows the binding affinities (Kd values) of DHA for the three types of muscles. Since the Kd values in the upper section of the table were obtained from binding experiments done in the absence of terbutaline, the values represent binding of DHA to both $\beta$1 and $\beta$2 adrenoceptors. There were no significant main effects or the effect of interaction of temperature and feed intake on receptor binding affinities ($P > 0.05$).

In the presence of terbutaline, the Kd values (Table 3.2, lower section) represent binding of DHA to $\beta$1-adrenoceptors only. DHA is selective for both $\beta$1 and $\beta$2 adrenoceptors. A high concentration of terbutaline (5μM) was used to occupy $\beta$2 adrenoceptors, leaving only $\beta$1 adrenoceptors for binding to DHA (Bylund et al. 1994). For the three muscles, there were no significant effects of temperature and feed intake on binding affinities of $\beta$1-adrenoceptors. However, for *semitendinosus,* the effect of interaction of temperature and feed intake was significant ($P = 0.05$).

The Bmax and Kd values for the three skeletal muscles with similar treatments were compared, in order to know whether there are differences between specific muscles. For

$\beta$1-adrenoceptors, only the Bmax values for *biceps femoris* were different from those of *gastrocnemius* and *semitendinosus* muscles ($P < 0.05$). There were no differences for the three muscles in terms of Bmax for $\beta$2-adrenoceptors ($P > 0.05$). Although differences exist for the Kd values (Table 3.2), no clear pattern was seen.

## 3.5 DISCUSSION

The three subtypes of beta-adrenoceptors ($\beta$1, $\beta$2 and $\beta$3) are closely related, with a high percentage of homology in amino acid sequence. But they are products of different genes (Bylund et al. 1994). All three activate adenylate cyclase through stimulatory guanine nucleotide regulatory protein, and can coexist within tissues (Galitzky et al. 1993) and on the same cells (del Monte et al. 1993). The results show that $\beta$1 and $\beta$2 adrenoceptors coexist in skeletal muscles (*biceps femoris, gastrocnemius* and *semitendinosus*) of sheep. However, Elfellah and Reid (1987) concluded that $\beta$-adrenoceptors in skeletal muscles (*gastrocnemius* and *soleus*) of pigs are predominantly, if not exclusively, of the $\beta$2 subtype. *Soleus* muscle of cat (Minneman et al. 1979), bovine *musculus trapezium* (Ijzerman et al. 1984) and *plantaris* muscles of rats (Kim et al. 1992) were found to have only the $\beta$2 subtype. But rat *soleus* muscle (Kim et al. 1992) were found to have both $\beta$1 and $\beta$2 adrenoceptors, similar to the results obtained for the skeletal muscles of sheep. The properties of a muscle are a reflection of the fibre type present (Elfellah and Reid 1987), but this can not explain the different adrenoceptor distribution obtained for the same type of muscles in different species. Other characteristics, for examples, species difference and cellular activity (Emorine et al. 1991), that cause differential receptor gene

expression are more likely to account for the differences. The three $\beta$-adrenoceptor subtypes have distinct genetic regulatory properties, thus allowing for modulation of adrenergic responsiveness of specific cells implicated in a particular physiological function. For example, the $\beta$2 contains two, $\beta$1 one, and the $\beta$3 no PKA-phosphorylation site (Lohse 1993). Correspondingly, there is a pronounced PKA-mediated downregulation of the $\beta$2, much less for the $\beta$1 and none for the $\beta$3 adrenoceptors. Such conditions could lead to more $\beta$1-adrenoceptors than $\beta$2-adrenoceptors in the plasma membranes, as observed in this study.

That the specific binding was saturable for DHA equilibrium binding (Fig. 3.1) is an indication of the presence of a finite number of binding sites. That the Scatchard plots for specific binding were linear, and the equilibrium binding data could not fit to a two-site model, implies that there was no negative co-operativity or the presence of multiple binding sites as was reported for binding of DHA to rat adipocyte membranes (Dax et al. 1982). These results are similar to those of Kim et al. (1992), Reddy and Engle (1979) and Reddy et al. (1979), who reported that DHA binding to rat skeletal muscle membranes was saturable and Scatchard analysis revealed that DHA binds to a single class of receptor sites.

The structures and functions of receptors and hormones, like those of all other proteins, are influenced by the environment, which includes temperature (Roth and Grunfeld 1985). The results indicate that cold temperature exposure and feed restriction increased the densities of $\beta$1-adrenoceptors, but no effects on $\beta$2-adrenoceptors and binding affinities in skeletal muscles of sheep. The interaction of temperature and feed

intake on receptor density was not significant. The main effect of feed intake was significant and more pronounced than that of temperature. For the three types of muscle (*biceps femoris, gastrocnemius and semitendinosus),* cold temperature increased the density of $\beta$1-adrenoceptors in animals with restricted feed. The physiological importance of this adaptation to cold can not be fully assessed on the basis of the existing data. Neither can a causal relationship between cold and $\beta$-adrenoceptor properties be inferred at this time, because no functional assessment of $\beta$1-adrenoceptors was made. However, several lines of evidence raise the possibility that an association between cold and increased density of $\beta$1-adrenoceptors may be physiologically relevant. Cold exposure induced an increase in oxidative metabolism in skeletal muscles (Lefaucheur et al. 1991), and Bmax of $\beta$-adrenoceptors correlates positively with oxidative capacity in skeletal muscles (Williams et al. 1984). These observations are consistent with a cold - induced increase in Bmax of $\beta$1-adrenoceptors, as obtained in this investigation. This increased receptor density in the cold for the feed restricted group could be responsible for the enhanced thermogenic response to epinephrine in cold-acclimated sheep as observed by Graham and Christopherson (1981). This speculation is made with the assumption that the biological responsiveness correlates with the binding data. The main effect of temperature resulted in slight increases in $\beta$1-adrenoceptors. It is not possible to say whether these increases are physiologically relevant, since no functional assessment was made.

The results indicate that feed restriction increased density of $\beta$1-adrenoceptors in skeletal muscles of sheep. This might have been due to upregulation of the receptors in

association with reduced release of catecholamines, since Shetty and Kurpad (1990) reported that fasting in rats suppresses the sympathetic nervous system. There is no report in the literature concerning the effect of feed restriction on density of $\beta$-adrenoceptors in skeletal muscles. Ostman et al. (1984) reported a decreased density of $\beta$-adrenoceptors in adipose tissue of fasted women, which was due to downregulation of receptors caused by high levels of circulating adrenaline. The differing response to feed restriction might be due to degree of restriction since the sheep were fasted for 24 hr only. The fact that a different species and tissue were studied, could also account for the different results. It should also be noted that fasted women would be losing weight, whereas the sheep used for the present study were gaining weight (chapter 2). The increased receptor density in the feed restricted groups could be responsible for the increased response to catecholamines for mobilization of substrates in support of energy metabolism (Deavers and Musacchia 1979).

Beta-2 adrenoceptors stimulation produces hypothermic effects in the cold, whereas $\beta$1-adrenoceptors stimulation has no apparent direct effect, but antagonizes the $\beta$2 elicited hypothermia when both subtypes are activated simultaneously by intra-peritoneal injection of agonists (Carlisle and Stock 1993). Rothwell et al. (1986) and Thorin et al. (1986) demonstrated that $\beta$1-adrenoceptors had thermogenic effects. Therefore, it would be an advantage for an animal in the cold to have more $\beta$1 than $\beta$2 adrenoceptors, and the present results show that this was the case for sheep in the cold environment. This suggestion has not been supported by the work of Fagher et al. (1986, 1988), however, which shows a major thermogenic role of the $\beta$2-adrenoceptors.

The results indicate that the Bmax values (for total $\beta$-adrenoceptors = beta-1 + beta-2) ranged from 35 to 70 fmol $mg^{-1}$ protein for *biceps femoris*; from 35 to 50 fmol $mg^{-1}$ for *gastrocnemius*, and from 30 to 50 fmol $mg^{-1}$ protein for *semitendinosus*. Reddy et al. (1979) reported Bmax values of 408 and 252 fmol $mg^{-1}$ protein for *extensor digitorum longus* and *soleus* muscles of rats, respectively. Elfellah and Reid (1987) reported 60 and 84 fmol $mg^{-1}$ protein for the *soleus* and *gastrocnemius* muscles of guinea pigs, respectively. Stadler et al. (1990) reported 40 to 50 fmol $mg^{-1}$ protein for *longissimus dorsi* of pigs, which are similar to the present results for sheep skeletal muscles. The Kd values, ranging from 1.5 to 4.5 nM, are also comparable to values obtained by Reddy et al. (1979) for rat *extensor digitorum longus* and *soleus* muscles. Stadler et al. (1990) reported a Kd value of 4.21 nM for *longissimus dorsi* of pigs. In all cases, [$^3$H]DHA was used as the radioligand. Since Kd value has a reciprocal relationship with receptor binding affinity, a high Kd value means a low receptor binding affinity. In this case, a high concentration of catecholamines would, therefore, be needed to effect responses. However, there were no significant main effects of temperature or feed intake on receptor binding affinity.

The results show that densities of $\beta$1-adrenoceptors in *biceps femoris* muscle are different from those of *gastrocnemius* and *semitendinosus* muscles, with the *biceps femoris* muscle having more $\beta$1-adrenoceptors than either *gastrocnemius* or *semitendinosus* muscles. *Gastrocnemius* and *semitendinosus* muscles had similar levels of $\beta$1-adrenoceptors. There were no differences obtained for densities of $\beta$2-adrenoceptors for the three types of skeletal muscles. On the basis of speed of their

contraction and histochemical profiles, skeletal muscles can be broadly classified into fast-twitch and slow-twitch types. These two types of skeletal muscles have been shown to respond differently to catecholamines in their contraction. Reddy et al. (1979) determined that catecholamines induced a greater percent of activation of adenylate cyclase and muscle contraction in slow-twitch muscle membrane than in fast-twitch muscle membrane. One would expect the slow-twitch muscle membrane to have more $\beta$-adrenoceptors, assuming the concept of 'spare receptors' where occupancy of a fraction of the receptors results in maximum biological responses (Roth and Grunfeld 1985) does not apply. But to the contrary, the $\beta$-adrenoceptors as determined by [$^3$H]DHA binding were greater in density in the fast-twitch muscle membranes (Reddy et al. 1979). The authors concluded that the enzyme and the receptors may be independently regulated. In some cases, the biological response to a ligand is proportional to the number of occupied receptors and a maximal response is elicited only when all the receptors are occupied. In other cases, only a portion of the receptor sites need be occupied to produce a maximal response, and the receptors not required to elicit a maximal response have been designated 'spare receptors'. For example, occupancy of a fraction of the receptors could lead to production of a submaximal concentration of cAMP, which results in a maximal biological response (Roth and Grunfeld 1985). The existence of spare receptors would, therefore, have unpredictable consequences for the comparison of binding data and physiological data for adrenergic receptors.

The three skeletal muscles used in this study are fast-twitch glycolytic or mixed fibre-types, and this can not explain the differences in the densities of $\beta$1-adrenoceptors as

observed in this study. However, according to their activities, the proportions of oxidative : oxidative-glycolytic : glycolytic fibre-types may be different. Aalhus (1988) showed that the percentage oxidative : oxidative-glycolytic : glycolytic fibre-types is different in different skeletal muscles of sheep, and that the percentages in *gastrocnemius* and *semitendinosus* muscles changed from 28:49:23 and 21:58:21 to 37:42:21 and 26:51:23 with 12 weeks of endurance exercises, respectively. These differences in proportions of fibre-types could account for the differences in oxidative capacities, and hence differences in densities of $\beta$1-adrenoceptors (as observed in this study), since the density of $\beta$-adrenergic receptors was found to correlate positively with oxidative capacity of the skeletal muscle (Williams et. al. 1984).

## 3.6 CONCLUSION

In conclusion, the results show that cold temperature exposure causes increased density of $\beta$1 -adrenoceptors in animals with restricted feed. Feed restriction increased the density of $\beta$1-adrenoceptors in the cold and warm environments. The effect of feed intake was more pronounced than that of temperature. The main effect of feed intake on $\beta$1-adrenoceptors was significant, whereas the main effect of temperature and the effect of interaction of temperature and feed intake were not significant. There was little or no effect on receptor binding affinity, and no effect on density of $\beta$2-adrenoceptors. Since the effectiveness of hormones is a function of their concentrations, binding affinities and their receptor densities (Roth and Grunfeld 1985), these results suggest that cold temperature exposure and feed restriction could increase metabolic responsiveness of

skeletal muscles to catecholamines through their actions on densities of beta-adrenoceptors. This may, in part, explain increased metabolic responsiveness to epinephrine (Graham and Christopherson 1981) in cold exposed sheep. However, the degree of the physiological changes expected due to the increased receptor number is not known since a functional assessment was not made.

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**Table 3.1**. Effects of temperature and feed intake on the density $(Bmax)^{Z,Y}$ of beta-2 adrenoceptors in skeletal muscles of lambs

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| *Biceps femoris* | 11.50 | 13.11 | 16.30 | 12.45 | 1.17 | 0.41 |
| *Gastrocnemius* | 12.17 | 10.52 | 12.45 | 10.24 | 0.89 | 0.33 |
| *Semitendinosus* | 11.21 | 10.10 | 10.95 | 10.35 | 1.00 | 0.31 |

[Z]Values are least squares means; N = 6.
[Y]Unit is $fmol.mg^{-1}$ protein
N/B: Main effects of temperature and feed intake are not significant ($P > 0.05$)
T*F indicate probabilities for interaction of temperature and feed intake.

**Table 3.2**. Effects of temperature and feed intake on DHA binding (Kd value)[Z,Y] to beta-adrenoceptors in skeletal muscles of lambs

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Beta-1 = beta-2 | | | | | | |
| *Biceps femoris* | 2.00 | 2.40 | 2.21 | 2.23 | 0.13 | 0.71 |
| *Gastrocnemius* | 1.58 | 1.66 | 1.51 | 1.73 | 0.19 | 0.89 |
| *Semitendinosus* | 3.04 | 2.42 | 2.39 | 3.08 | 0.34 | 0.78 |
| Beta-1 only | | | | | | |
| *Biceps femoris* | 2.11 | 2.41 | 1.93 | 2.62 | 0.17 | 0.23 |
| *Gastrocnemius* | 3.01 | 3.34 | 2.94 | 3.44 | 0.23 | 0.31 |
| *Semitendinosus* | 3.84 | 4.31 | 3.77 | 4.38 | 0.39 | 0.05 |

[Z]Values are least squares means; N = 6.
[Y]Unit is nM
N/B: Main effects of temperature and feed intake are not significant (P > 0.05).

**Figure 3.1** Saturation binding curve for equilibrium binding of [$^3$H]dihydroalprenolol ([$^3$H]DHA) to beta-1 and beta-2 adrenergic receptors in ovine skeletal muscle membrane (values are means ± SEM of replicate tubes in the single assay for one animal).

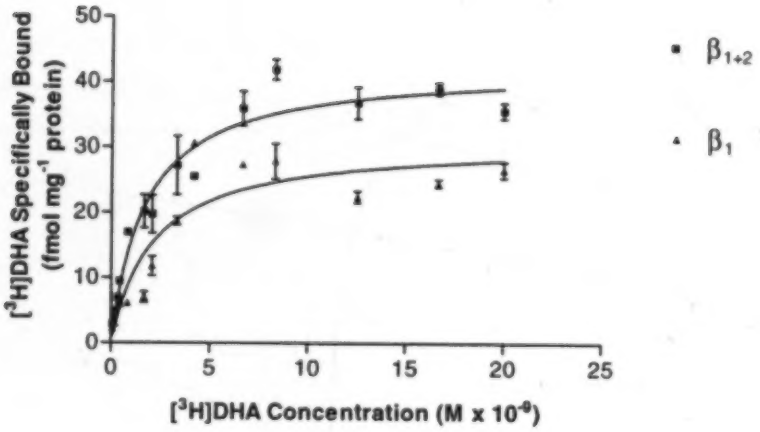
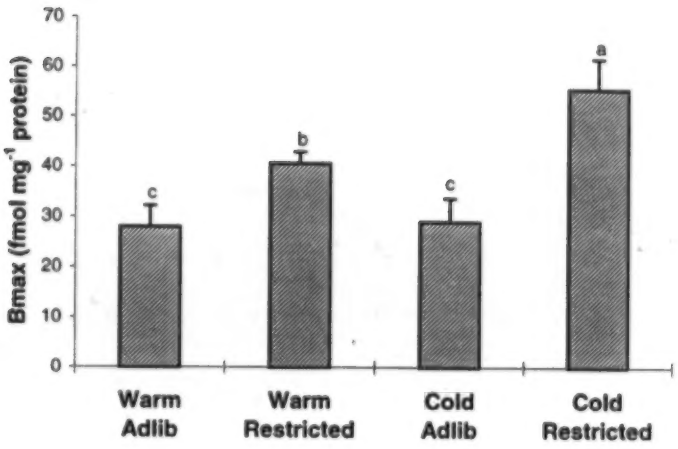


**Figure 3.2** Density (Bmax) of beta-1 adrenergic receptors in *biceps femoris* muscle membranes of lambs raised in the cold and warm environment and fed at restricted or *ad libitum* level (values are means ± SEM; N = 6. Different superscripts indicate a significant difference; $P < 0.05$).




The table below shows the main effects[Z] of temperature (T) and feed intake (F) and their interaction (T*F) on the density of beta-1 adrenoceptors in *biceps femoris* muscle

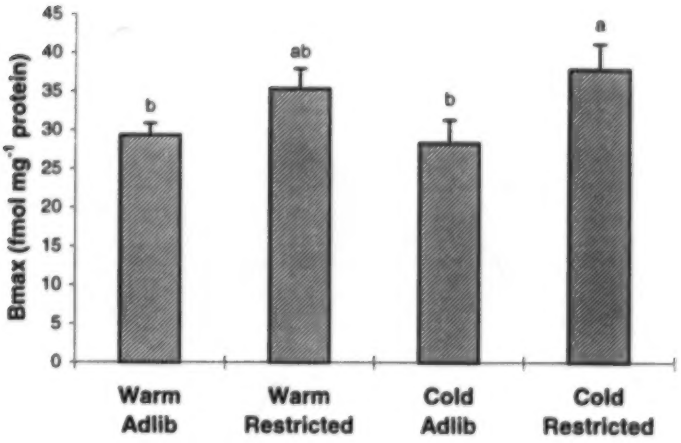
| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| *Biceps femoris* | 34.35 | 42.28 | 28.51d | 48.12c | 3.18 | 0.13 |

[Z]Values are least squares means; N = 6

c, d indicate significant main effect of feed intake ($P < 0.001$)

N/B: Main effect of temperature is not significant ($P > 0.05$)

**Figure 3.3** Density (Bmax) of beta-1 adrenergic receptors in *gastrocnemius* muscle membranes of lambs raised in the cold and warm environment and fed at restricted or *ad libitum* level (values are means ± SEM; N = 6. Different superscripts indicate a significant difference; $P < 0.05$).




The table below shows the main effects[Z] of temperature (T) and feed intake (F) and their interaction (T*F) on the density of beta-1 adrenoceptors in *gastrocnemius* muscle

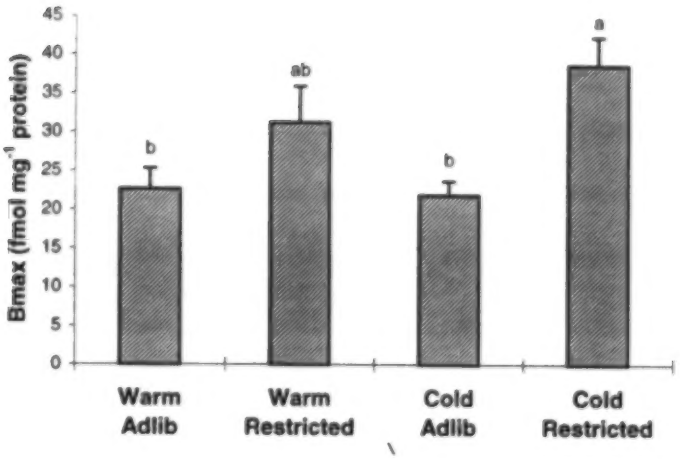
| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| *Gastrocnemius* | 32.35 | 33.13 | 28.86d | 36.62c | 1.89 | 0.51 |

[Z]Values are least squares means; N = 6

c, d indicate significant main effect of feed intake ($P < 0.01$)

N/B: Main effect of temperature is not significant ($P > 0.05$)

**Figure 3.4.** Density (Bmax) of beta-1 adrenergic receptors in *semitendinosus* muscle membranes of lambs raised in the cold and warm environment and fed at restricted or *ad libitum* level (values are means ± SEM; N = 6. Different superscripts indicate a significant difference; $P < 0.05$).




The table below shows the main effects[Z] of temperature (T) and feed intake (F) and their interaction (T*F) on the density of beta-1 adrenoceptors in *semitendinosus* muscle

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| *Semitendinosus* | 26.94 | 30.24 | 22.25d | 34.92c | 2.38 | 0.23 |

[Z]Values are least squares means; N = 6

c, d indicate significant main effect of feed intake ($P < 0.001$)

N/B: Main effect of temperature is not significant ($P > 0.05$)

# CHAPTER 4

## Effects of Temperature and Plane of Nutrition on Beta-Adrenergic Receptors in Heart, Kidney and Liver of Lambs

## 4.1 INTRODUCTION

Physiological and pharmacological data suggest that beta-adrenoceptors, originally classified by Ahlquist (1948), can be classified into three subtypes, beta-1, beta-2 and beta-3 adrenoceptors. These three subtypes of beta-adrenoceptors (β-adrenoceptors) may co-exist in the same tissue (Galitzky et al. 1993) and in the same cells (del-Monte et al. 1993; Homburger et al. 1981). Beta-1 and β2-adrenoceptors coexist in heart (Robberecht et al. 1983) and in kidney (Snavely et al. 1982), and β2- and β3- adrenoceptors coexist in liver cells (Ruffolo Jr. 1994).

The three beta-receptor subtypes mediate physiological responses to endogenous catecholamines, epinephrine and norepinephrine (Christopherson et al. 1995) and synthetic sympathomimetic agents (Arch and Kaumann 1993). They are products of different genes but closely related, with a high percentage of homology in amino acid sequence (Bylund et al. 1994). They are linked to guanine nucleotide (stimulatory) regulatory protein, which activates adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) inside the cells. The cAMP activates protein kinase A, which phosphorylates proteins, leading to different physiological responses to catecholamines and other adrenergic agents (Ruffolo Jr. 1994). For example, adrenergic responsiveness is

known to be modified by environmental and nutritional factors. Cold temperature exposure increases activities of sympatho-adreno-medullary system, leading to increased release of endogenous adrenergic agents (Christopherson et al. 1978, Sasaki and Weekes 1984). In rats, fasting suppresses and over-feeding stimulates the sympathetic nervous system (Shetty and Kurpad 1990). It is also possible for tissue responsiveness to be modulated at any level or combination of levels in the signal transduction pathway for the beta-adrenergic receptors. This modulation may include changes in beta-adrenoceptor density and / or binding affinity.

Radioligand binding studies have demonstrated alterations in the densities of β-adrenoceptors in a number of pathological and physiological conditions (Kawai and Arinze 1983, Bao 1990, Watanabe et al. 1991). Cold exposure and feed restriction have effects on densities of β1-adrenoceptors in skeletal muscles of sheep (Chapter 3, Ekpe et al. 1997). The purpose of the present work was to investigate the presence and relative abundance of β1- and β2- adrenoceptors in heart, kidney and liver tissues of wether lambs, and to determine the effects of cold temperature exposure and feed restriction on density and binding affinity of these beta-adrenergic receptor subtypes.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Animals and Management

Animals and treatments are the same as in chapters 2 and 3. Twenty four Suffolk-cross, six months old wether lambs, randomly divided into four groups, WA, WR, CA and CR

were housed in separate pens (180 x 90 x 90 cm). The lambs were exposed to either warm (W; 23 ± 2 °C) or cold (C; 0 ± 2 °C) temperatures. In each environment, the lambs were fed experimental diets of alfalfa pellets (17 % crude protein, 9.08 MJ ME $Kg^{-1}$ DM; see Appendix 3 for feed composition) at an *ad libitum* (A) or a restricted (R) level. The levels of feed restriction were 1.35 x maintenance in the warm environment, and 130 % of the restricted level of the warm environment in the cold environment. These levels of restriction, based on published information on heat production and total body insulation of shorn sheep (NRC 1975, Christopherson and Young 1981, McBride and Christopherson 1984 a, b) were chosen to allow for a reasonable weight increase. Water and cobalt-iodized salt were supplied *ad libitum*. All lambs were shorn to a fleece depth of approximately 1 cm, and re-shorn at 3-week intervals.

The care and handling of the wethers used in this study conformed to the guidelines established by the Canadian Council on Animal Care. The lambs were initially adapted to treatments for 28 d before the experiment. The experiment was divided into three periods of five weeks each. In periods one and three, the treatments described above were imposed. During period two, WR and CR groups were removed from the experiment and these animals were fed *ad libitum* in a thermoneutral environment to prevent major declines in body condition prior to period three. This action was based on visual assessment of the body conditions of the animals. The lambs were slaughtered at the end of period three, and hearts, kidneys and livers were harvested, wrapped in aluminium foil and frozen in liquid nitrogen, and then stored frozen at -72 °C. All animals were fasted for

24 hr before slaughtering to reduce gut fills and facilitate the evisceration and tissue sampling process.

### 4.2.2 Membrane Preparation

Membrane preparation was performed according to methods of Ohlendieck et al. (1991). Thirty g of the frozen tissue was cut into pieces and homogenized three times (30 seconds, with 2 min intervals) in 210 mL of buffer A (20 mM tetrasodium pyrophosphate, 20 mM sodium phosphate, 1 mM magnesium chloride, 0.303 M sucrose, 0.5 mM ethylenediaminetetraacetic acid; EDTA, 76.8 nM aprotinin, 1.1 μM leupeptin, 0.7 μM pepstatin A, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM phenylmethylsulfonyl fluoride; PMSF, pH 7.0) using a Waring blender. The homogenate was centrifuged using a JA-10 rotor at 14,000 x g for 15 min and filtered through 6 layers of cheese cloth. The pellet was homogenized again in 150 mL of buffer A, centrifuged and filtered. The combined supernatant was centrifuged using a JA-14 rotor at 30,000 x g for 30 min and filtered. Solid KCl was added to the filtrate to a concentration of 0.6 M, and the filtrate centrifuged in 60 Ti-rotor at 142,000 x g for 35 min. All the centrifugation was done at a temperature of 4 °C and all glasswares were placed on ice. The pellet was resuspended in 4 mL of buffer B (0.303 M sucrose, 20 mM tris-maleate, 0.6 M KCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0), and stored in 1 mL aliquots at -72 °C. These aliquots were used for determination of protein content and for radioligand binding assays. The protein concentration of the membrane extracts, in mg

$mL^{-1}$, was determined by Bradford's test (Bradford 1976), using bovine serum albumin as a standard.

### 4.2.3 Radioligand Binding Assay

Radioligand binding assays were performed according to methods of Mersmann and McNeel (1992) with slight modifications. Membrane extracts (1 mg $mL^{-1}$ protein content) and 12 concentrations (0.08 - 20 nM) of tritiated dihydroalprenolol ([$^3$H]DHA, specific activity 57.0 Ci $mmol^{-1}$, New England Nuclear) were incubated at 25 °C for 30 min in buffer B, at a final volume of 150 µL. The final measurement gave the total binding. Nonspecific binding was determined using the same concentrations of [$^3$H]DHA as mentioned above in the presence of $10^{-5}$ M (±)-propranolol. Incubation was stopped by addition of 5 mL ice-cold buffer B to each tube, followed by rapid vacuum filtration through Whatman GF/B glass fibre (pre-soaked in 0.5 % polyethylenimine to reduce non-specific binding). The filters were washed twice with 5 mL of ice-cold buffer B and the filter-bound radioactivity was measured by liquid scintillation spectrometry.

Specific binding was defined as total binding minus nonspecific binding. Since DHA is a selective antagonist for both β1 and β2 adrenoceptors (Bylund et al. 1994), the specific binding indicates binding of [$^3$H]DHA to both receptor subtypes. A third binding experiment was done for each concentration of [$^3$H]DHA in the presence of 5 µM terbutaline (Bylund et al. 1994), with the assumption that the terbutaline binds to all β2-adrenoceptors, and the specific binding represents the binding to β1-adrenoceptors.

Binding to β2-adrenoceptors was then estimated by difference. Duplicate binding analysis was performed for each concentration of ligand.

## 4.3 DATA ANALYSIS

The binding data obtained was subjected to curve fitting procedures, using the non-linear regression analysis of Graphpad Prism (Graphpad 1995), to obtain density (maximum binding capacity, Bmax) of β1 and β2 adrenoceptors, and binding affinity (Kd) of the receptors. The results, calculated as means ± SEM, were evaluated as a 2 X 2 factorial, using GLM procedures of SAS program (SAS 1990). The model included a two-way analysis of variance and a Duncan multiple range test. The differences among means were compared using a Duncan multiple range test. The sources of variations were temperature, feed intake and the interaction between them.

## 4.4 RESULTS

Figure 4.1 shows a typical saturation binding curve of [$^3$H]DHA. The specific binding was saturable for DHA equilibrium binding, and all the Scatchard plots for specific binding were linear and fit with a correlation coefficient > 0.88. The equilibrium binding data could not fit to a two-site model, and there was no evidence for curvilinear Scatchard plots.

### 4.4.1 Heart

The main effect of feed intake on density of β1 adrenoceptors was significant ($P < 0.01$). The effect of interaction of temperature and feed intake was also significant ($P < 0.05$). However, the main effect of temperature was not significant ($P > 0.05$). [$^3$H]DHA saturation binding to the heart membranes indicates a higher density (Bmax) of β1 adrenoceptors for the CA group compared with the other groups (Fig. 4.2). In the cold environment, Bmax for CR was lower than that of CA ($P< 0.01$), and similarly, the Bmax for WR was lower than that of WA ($P< 0.05$) in the warm environment. When the two environments were compared, the Bmax for CA was higher than, but not significantly different from that of WA ($P > 0.05$), whereas the Bmax for CR was lower than that of WR ($P< 0.01$).

In the case of β2-adrenoceptors (Table 4.1), the main effect of feed intake on Bmax of β2 adrenoceptors was significant ($P < 0.05$). Cold exposure increased the density of β2 adrenoceptors, but this increase was not significant ($P > 0.05$). In addition, the interaction of temperature and feed intake did not have a significant effect in the heart ($P > 0.05$). The binding affinities (Kd) were approximately similar in all groups for total β-adrenoceptors (β1 + β2) and β1 adrenoceptors (Table 4.2). In the absence of terbutaline, the Kd values represent binding affinities for both β1- and β2- adrenoceptors, herein referred to as 'total beta-adrenoceptors' (upper section of the table). But in the presence of terbutaline, the binding affinities are for β1- adrenoceptors only (lower section of the table). Neither temperature nor feed intake had effects on binding affinities. However, the

effect of interaction of temperature and feed intake on binding affinities of β1-adrenoceptors was close to significance (P = 0.08).

### 4.4.2 Kidney

The main effect of feed intake on density of β1-adrenoceptors in the kidney was significant (P < 0.01), while the effect of interaction of temperature and feed intake was close to significance (P = 0.07). [$^3$H]DHA saturation binding to the kidney membranes indicated a pattern opposite to that of the heart, with a lower density of β1 adrenoceptors for the CA group compared with the other groups (Fig. 4.3). In the cold environment, Bmax for CR is higher than the Bmax for CA (P< 0.01), whereas, in the warm environment, Bmax for WR is higher than, but not significantly different from that for WA (P > 0.05). Comparison of the two environments showed that the Bmax were similar between CA and WA (P > 0.05), but Bmax for CR was higher than that for WR (P< 0.05).

In the case of the Bmax for β2 adrenoceptors in the kidney membranes, comparison did not reveal any differences between the two groups in each environment (Table 4.1), or between the two environments (P > 0.05). There was no effect of interaction of temperature and feed intake on Bmax of β2 adrenoceptors. The Kd values followed a similar pattern as Bmax for β1 adrenoceptors (Table 4.2). For both total β-adrenoceptors and β1- adrenoceptors, the main effects of temperature and feed intake on binding affinities were significant (P < 0.01). The effect of interaction of temperature and feed

intake was significant ($P < 0.05$) for total β- adrenoceptors and close to significance ($P = 0.08$) for β1- adrenoceptors.

### 4.4.3 Liver

[$^3$H]DHA saturation binding to the liver membranes indicated a higher density (Bmax) of β1 adrenoceptors for the CR group than for the CA group in the cold environment ($P< 0.05$). However, there was no difference ($P > 0.05$) due to feeding level, WR versus WA, in the warm environment (Fig. 4.4). Comparison of the two environments did not show any differences between CA and WA, or between CR and WR ($P > 0.05$). The main effect of feed intake on density of β1- adrenoceptors in the liver was significant ($P < 0.01$). The effect of interaction of temperature and feed intake was close to significance ($P = 0.06$). However, the main effect of temperature on the density of β1- adrenoceptors in the liver was not significant ($P > 0.05$).

The Bmax for β2-adrenoceptors in the liver were similar in all groups. Similar to the results for the kidney, comparison did not reveal any differences between the two groups in each environment (Table 4.1) or between the two environments ($P > 0.05$). There was no effect of interaction of temperature and feed intake on Bmax of β2 adrenoceptors ($P > 0.05$). In the case of the binding affinities (Table 4.2), the main effect of temperature was significant ($P < 0.001$) for total β- adrenoceptors (upper section of the table) and for β1- adrenoceptors (lower section of the table). In both cases, the main effect of temperature and the effect of interaction of temperature and feed intake were not significant ($P > 0.05$).

### 4.4.4 Heart and Kidney Weights

The main effect of feed intake on organ weight was significant ($P < 0.001$). The main effect of temperature and the interaction of temperature and feed intake were not significant, $P > 0.05$ (Table 4.3) In the cold environment, CA had a greater heart weight than CR ($P < 0.001$). Similarly, WA had a greater heart weight than WR ($P < 0.001$) in the warm environment. Comparism of the two environments revealed that cold exposure caused increased heart weight in the *ad libitum* fed group, but the difference was not significant ($P > 0.05$). The weights of the left kidneys of the wether lambs followed a similar pattern as the weights of the hearts, with feed restriction leading to reduced kidney weights ($P < 0.001$), and cold exposure causing slight increases in the left kidney weights.

## 4.5 DISCUSSION

Beta-adrenoceptors are members of the guanine nucleotide regulatory protein (G-protein) coupled receptor family (Caron and Lefkowtz 1993) which includes the receptors for a variety of neurotransmitter hormones and sensory stimuli. Physiological and pharmacological data indicate that $\beta$1 and $\beta$2 adrenoceptors may coexist in the same tissue (Galizky et al. 1993). The present study has demonstrated that both receptor subtypes coexist in heart, kidney and liver tissues of sheep, but in different proportions. In chapter 3, it was determined that skeletal muscles of sheep also contain the $\beta$1- and $\beta$2-subtypes of $\beta$-adrenoceptors (also Ekpe et al. 1997).

Binding of [$^3$H]DHA to these receptors in all three tissues was saturable, indicating the presence of a finite number of binding sites. The Scatchard plots for specific binding were linear, and the equilibrium binding data could not fit to a two-site model, suggesting that there was no negative co-operativity or the presence of multiple binding sites. Similar results were obtained for skeletal muscles of sheep (Chapter 3, Ekpe et al. 1997). Rothwell and co-workers (1985) had reported similar results for rat heart, and Gavendo et al. (1980) and Snavely et al. (1982) reported similar results for rat kidney. In contrast to the above, binding of [$^3$H]DHA to rat kidney $\beta$-adrenoceptors (Woodcock and Johnston 1980a) yielded curvilinear Scatchard plots, suggesting the presence of multiple classes of binding sites with negative co-operativity.

### 4.5.1 Heart

Cold temperature exposure increases activities of the sympatho-adreno-medullary system, with increased release of endogenous catecholamines (Sasaki and Weekes 1984), leading to increased responses. Increased receptor number is one of the possibilities that could increase tissue responsiveness to catecholamines. The results indicate a higher $\beta$1 receptor number for the CA group than for any other group, but the CR group, which was also in the cold environment had the least number of $\beta$1 adrenoceptors. Cold temperature exposure had a significant effect only with feed restriction, but the main effect of temperature on the density of $\beta$1-adrenoceptors was not significant. Feed restriction had significant effects in both cold and warm environments. Feed restriction reduced the

number of β1 adrenoceptors, cold temperature reduced and increased β1 adrenoceptors with and without feed restriction, respectively. On the other hand, temperature and feed intake did not affect the density of β2-adrenoceptors or receptor binding affinities. In a situation where the biological responsiveness can be correlated with binding data, the decrease in β1-adrenoceptors by feed restriction is expected to result in significant decreases in rate of heart beat and force of contraction.

Previous studies indicated beta-adrenoceptor densities of about 164 - 209 fmol $mg^{-1}$ protein in fetal sheep heart (Tseng et al. 1995), 3600 fmol $mg^{-1}$ protein in dog heart (Baker and Potter 1980), 74.6 fmol $mg^{-1}$ protein in dog heart (Muntz etal. 1993), 50 fmol $mg^{-1}$ protein in rabbit heart (Borsodi et al. 1983), 49 - 90 fmol $mg^{-1}$ protein in pig heart (Stadler et al. 1990), 19.8 fmol $mg^{-1}$ protein in rat heart (Lu and Barnett 1990), and 43 - 88 fmol $mg^{-1}$ protein in human heart (Bristow et al. 1986). In the case of the present study, the results indicate β-adrenoceptor number of about 89 - 215 fmol $mg^{-1}$ protein in sheep, and this range is similar to that reported in fetal sheep but somewhat higher than densities reported for dog, rabbit, pig, and human heart tissue. Differences in the number of β-adrenoceptors in heart could be due to species differences, sex, age, nutritional and physiological states of the animals.

It is now considered that cardiac tissue usually contains a majority of β1-adrenoceptor subtype. Hedberg et al. (1980) reported an apparent homogenous population of β1-adrenoceptor (100 %) in ventricles of cat and guinea pig, whereas the atria of both species contained both β1- and β2- adrenoceptors in a ratio of approximately 3:1. Other results are 88 - 95 % β1, 5 - 12 % β2 (present study), 83 % β1 adrenoceptor in rat heart

(Minneman et al. 1979), 60 % β1-adrenoceptor in rat heart (Rothwell et al. 1985), and 75 % β1-adrenoceptor in human heart (Bjornerheim et al. 1991). Bristow et al. (1986) reported 60 % β1-adrenoceptor in failing human ventricle and 77 % β1-adrenoceptor in non-failing human ventricle, with selective downregulation of β1-adrenoceptor in failing ventricle. In contrast, Robberecht et al. (1983) reported a much higher proportion of β2-adrenoceptor in human auricles (50 % β1 : 50 % β2). The above reports indicate variations due to tissue type (ventricle or auricle), species and physiological conditions of the animals used. For example, Roth and Grunfeld (1985) indicated that the type and number of receptors in a cell are dependent on the cell's genetic make-up, activity and development program.

The Kd values of 2.11 - 4.35 nM for binding of DHA to membranes of sheep heart ventricle agree with the Kd values reported by other authors; 3.5 nM for fetal sheep heart (Tseng et al. 1995), 3.29 - 5.03 nM for pig heart (Stadler et al. 1990) and 2.2 nM for rat heart (Rothwell et al. 1985). Cold exposure and feed restriction did not have any effects on the receptor binding affinities in the heart.

### 4.5.2 Kidney

In mammalian kidney, beta-adrenoceptors regulate numerous functions, including renal blood flow, renin release, erythropoietin production and certain tubular functions (Insel and Snavely 1981). The present study indicates that feed intake have significant effects on density of β1-adrenoceptors of ovine kidney. Feed restriction increased the density of β1-adrenoceptors in both cold and warm environments, while cold temperature exposure

only increased the density of β1-adrenoceptors in animals with feed restriction. The functional significance or the implication of these changes is that cold temperature exposure and feed restriction could increase metabolic responsiveness of the kidney to catecholamines. The increased responses could affect any of the kidney functions such as glycogenolysis, renin release, erythropoietin production or maintenance of ionic and water balance. Neither temperature or feed intake influenced the density of β2-adrenoceptors in ovine kidney.

Of the total number of *β*-adrenoceptors of about 35 - 48 fmol $mg^{-1}$ protein, β1- and β2- adrenoceptors are 86 % and 14%, respectively. Snavely et al. (1982) have reported that rat renal cortex contains 46 fmol $mg^{-1}$ protein of *β*-adrenoceptors, of which β1- and β2- adrenoceptors are 70 % and 30 %, respectively. In another report by Snavely et al. (1985a), the concentration of *β*-adrenoceptors in rat renal cortex was 41 fmol $mg^{-1}$ protein, and β1 and β2 were 60 % and 40 %, respectively. The same authors (1985b) found about 27 fmol $mg^{-1}$ protein of *β*-adrenoceptors in rat renal cortex, with 75 % β1-adrenoceptors and 25 % β2-adrenoceptors. They concluded that the number of *β*-adrenoceptors depends on the circulating catecholamines in blood plasma, and also suggested that β1- and β2- adrenoceptor numbers vary because of their differential regulation. The variation in number of *β*1- and *β*2- adrenoceptors, as observed in this study, may be due to the genetic make-up of the kidney cells, differential downregulation of receptors or different activities of the kidney cells.

The present results also indicate that temperature and feed intake significantly affect the binding affinities of DHA to the *β*-adrenoceptors in the kidney membranes. Feed

restriction caused decreased binding affinity (increased Kd values) in both cold and warm environments, and cold temperature exposure decreased binding affinity with feed restriction. In this case, a high concentration of catecholamines would be needed to effect biological responses. Therefore, this reduction in receptor binding affinity could result in decreased kidney responsiveness to catecholamines. The Kd values obtained in this study are similar to the Kd values of 1.5 - 4.4 nM for binding of DHA to rat kidney membranes (Woodcock and Johnston 1980 a; b), although their results, in contrast to the present study, indicated the presence of multiple classes of binding site (Bmax = 33.3 fmol $mg^{-1}$ protein) with negative co-operativity.

### 4.5.3 Liver

Ruffolo Jr. et al. (1994) reported that β2 and β3 adrenoceptors stimulate glycogenolysis in liver. McDowell and Annison (1991) had earlier reported that glycogenolysis in the liver is under the control of *β*2-adrenoceptors. Kawai and Arinze (1983) showed that the potency order for the displacement of bound [$^3$H]DHA to purified rabbit liver (Bmax = 434 fmol $mg^{-1}$ protein, Kd = 1.39 nM), isoproterenol > epinephrine > norepinephrine, is similar to glycolytic effects of catecholamines in the liver. They concluded that the *β*-adrenoceptors in the liver are predominantly, if not exclusively, of β2 subtype. The present study shows that ovine liver contains approximately 15.0 fmol $mg^{-1}$ protein β1-adrenoceptor and 4.0 fmol $mg^{-1}$ protein β2-adrenoceptors (Kd = 1.3 - 5.9 nM). Temperature did not have an effect on densities of either β1- or β2- adrenoceptors, but

cold temperature exposure reduced the binding affinities. Therefore, a high concentration of catecholamines would be required to effect biological responses in the liver of animals raised in the cold.

Feed intake did not have effect on density of β2-adrenoceptors or receptor binding affinities, but feed restriction increased the density of β1 adrenoceptors in the cold. The increased *β*1-adrenoceptor number by feed restriction could increase such functions as glycogenolysis, glycolysis and amino acid and lipid metabolism. Such mechanisms could result in mobilization of substrates in support of energy metabolism. The combination of cold and feed restriction increased *β*1-adrenoceptor densities in the liver. Graham and Phillips (1981) reported that epinephrine produced a larger increase in plasma glucose concentration in cold acclimated sheep. This could mean increased *β*-adrenergic responsiveness of the liver when cold and feed restriction coincide as suggested by the present study.

The low concentration of *β*-adrenoceptors agree with the data for rat liver, Bmax = 19 fmol $mg^{-1}$ protein (Bendeck and Noguchi 1985) and rat liver *β*2-adrenoceptors, Bmax = 8.1 fmol $mg^{-1}$ protein; Kd = 0.18 nM (Ishac et al. 1992). In contrast, Kawai and Arinze (1983) and Wolfe et al. (1976) reported 75 fmol $mg^{-1}$ protein and 60 fmol $mg^{-1}$ protein of *β*-adrenoceptors, respectively, in rat liver. Bendeck and Noguchi (1985) observed that newborn rat liver contains more beta- and less alpha- adrenoceptors than adult liver. And investigations led them to conclude that the change in glycogen phosphorylase activation from beta- to predominantly alpha- adrenergic mechanisms seen with maturation may be related to changes in adrenoceptor density. The above reports indicate that the number

and subtype of $\beta$-adrenoceptors in the liver vary, depending on species, age and physiological states of the animals.

### 4.5.4 Heart and Kidney Weights

Cold temperature exposure resulted in slight increases in heart and left kidney weights. In contrast, feed restriction caused significant reduction in the weights of the hearts and left kidneys. The reduction in organ weight may be related to the reduced average daily gain in animals with feed restriction, as observed in chapter two. Murphy and Loerch (1994) indicated that reducing the size of metabolically active organs (eg., heart and kidney) is one of the mechanisms of reducing maintenance energy expenditures. Such mechanism may be important to ruminant animals during periods of food scarcity. The effects of feed restriction on the heart weights are similar to the effects of feed restriction on densities of $\beta$1-adrenoceptors in heart membranes. A linear regression analysis indicated that there is a positive relationship between the density of $\beta$1-adrenoceptors in heart membrane and the heart weight. In contrast to the above, there is a negative relationship between the density of $\beta$1-adrenoceptors in the kidney membrane and the left kidney weight.

**Equation 4.1**: $Y = -49.8 + 0.9\ X$ ( for heart; $r^2 = 0.44$).

**Equation 4.2**: $Y = 48.8 - 0.2\ X$ ( for kidney; $r^2 = 0.25$).

Where; Y = density of $\beta$1-adrenoceptors,

X = weight of organ.

However, this relationship disappeared in both cases when the ratio of organ weight to body weight was used. The physiological importance of this relationship between the density of $\beta$-adrenoceptors and the organ weight can not be fully assessed on the basis of the existing data. Neither can a causal relationship between the density of $\beta$-adrenoceptors and the organ weight be inferred. In the case of the heart, there could be an increase in heart weight in response to increased work load due to increased pressure against which the heart muscle must contract (Guyton 1976). Since activation of $\beta$-adrenoceptors in the heart results in stimulation of frequency (chronotropic effect on sino-atrial node) and force (inotropic effect on myocardial cells) of contraction, the relationship described above may have a physiological basis. A large heart would require a larger force of contraction, and hence, a greater number of $\beta$-adrenoceptors, as observed in the present study. The increased $\beta$1-adrenoceptors in smaller kidneys may be in support of metabolism in the cold and during feed restriction. More research is required to determine whether the relationship between the density of $\beta$-adrenoceptors and the organ weight does have any physiological relevance.

## 4.6 CONCLUSION

The present study confirms that ovine heart, kidney and liver contain both $\beta$1- and $\beta$2-adrenoceptors. Data show that membranes from these tissues contain a single class of nonco-operative binding sites for [$^3$H]DHA. Temperature and feed intake had different effects in heart, kidney and liver, with cold temperature exposure reducing $\beta$1

adrenoceptor density in heart and increasing its densities in kidney and liver with feed restriction. Feed restriction reduced $\beta$1-adrenoceptor density in heart, and increased $\beta$1-adrenoceptor densities in kidney and liver. In terms of relative densities of $\beta$1-adrenoceptors, only the heart tissue had a pattern that differed from the previous findings on skeletal muscles of sheep (Chapter 3, Ekpe et al. 1997). Since density of $\beta$-adrenoceptors correlates positively with oxidative capacity in skeletal muscles (Williams et al. 1984), and the effectiveness of hormones is a function of their concentrations, binding affinities and their receptor densities (Roth and Grunfeld 1985), these results imply that cold temperature exposure and feed restriction could reduce (in heart) and increase (in kidney and liver) metabolic responsiveness of tissues to catecholamines. This conclusion is made with an assumption that biological responsiveness correlates with binding data. But this conclusion does not apply to cases involving 'spare receptors' where occupancy of a fraction of the receptors results in maximum biological responses (Roth and Grunfeld 1985). Cold temperature and feed restriction reduced receptor binding affinities in kidney and liver, and a high concentration of catecholamines would, therefore, be needed to effect responses. Since the concentration of catecholamines remains high in a cold environment (Sasaki and Weekes 1984), the change observed in receptor density is likely more important than that of the change in binding affinity.

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**Table 4.1**. Effects of temperature and feed intake on the density (Bmax)[Z,Y] of beta-2 adrenoceptors in heart, kidney and liver tissues of lambs

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Heart | 8.68 | 10.15 | 11.86c | 6.98d | 1.38 | 0.71 |
| Kidney | 5.79 | 5.43 | 5.33 | 5.90 | 0.69 | 0.47 |
| Liver | 3.54 | 4.35 | 3.91 | 3.98 | 0.61 | 0.64 |

[Z] Values are least squares means; N = 6.

[Y] Unit is fmol.mg$^{-1}$ protein

c, d indicate significant main effect of feed intake ($P < 0.05$)

T*F indicate probabilities for interaction of temperature and feed intake

**Table 4.2**. Effects of temperature and feed intake on DHA binding (Kd value)[Z,Y] to beta-adrenoceptors in heart, kidney and liver tissues of lambs

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Beta-1 + beta-2 | | | | | | |
| Heart | 2.85 | 4.04 | 2.98 | 2.91 | 0.59 | 0.60 |
| Kidney | 2.31b | 4.02a | 1.69d | 4.63c | 0.30 | 0.03 |
| Liver | 1.93b | 3.77a | 2.47 | 3.23 | 0.34 | 0.93 |
| Beta-1 only | | | | | | |
| Heart | 2.69 | 2.32 | 2.34 | 2.70 | 0.31 | 0.08 |
| Kidney | 2.21b | 3.69a | 1.95d | 3.95c | 0.33 | 0.08 |
| Liver | 3.59b | 5.73a | 4.47 | 4.85 | 0.25 | 0.67 |

[Z] Values are means; N = 6.

[Y] Unit is nM

a, b indicate significant main effect of temperature ($P < 0.001$)

c, d indicate significant main effect of feed intake ($P < 0.001$)

T*F indicate probabilities for interaction of temperature and feed intake

**Table 4.3**. Effects of temperature and feed intake on the weights[Z,Y] of heart and left kidneys of lambs

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Heart | 223.4 | 235.6 | 268.0c | 191.2d | 6.93 | 0.18 |
| Kidney | 81.3 | 87.6 | 103.6c | 65.4d | 3.85 | 0.41 |

[Z] Values are means; N = 6.

[Y] Unit is g

c, d indicate significant main effect of feed intake ($P < 0.001$)

T*F indicate probabilities for interaction of temperature and feed intake

**Figure 4.1** Saturation binding curve for equilibrium binding of [$^3$H]dihydroalprenolol ([$^3$H]DHA) to beta-1 and beta-2 adrenergic receptors in heart membrane (values are means ± SEM of replicate tubes in the single assay for one animal).

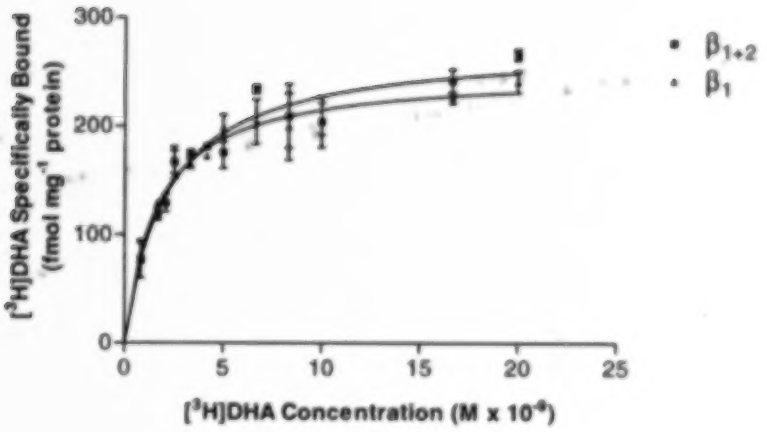
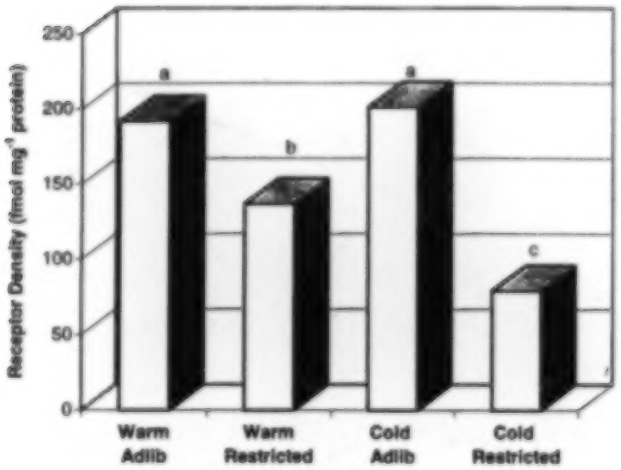


**Figure 4.2** Density (Bmax) of beta-1 adrenergic receptors in heart muscle membranes of lambs raised in the cold or warm environment and fed at restricted or *ad libitum* level (values are means; N = 6. Different superscripts indicate significant difference, $P < 0.05$).

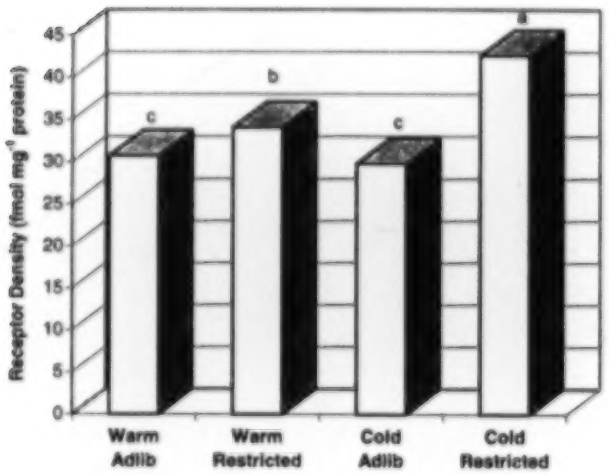



The table below shows the main effects of temperature and feed intake and their interaction (T*F) on density of beta-1 adrenoceptors in heart

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Heart | 164.31 | 140.25 | 196.55c | 108.01d | 11.15 | 0.04 |

Values are least squares means; N = 6
c, d indicate significant main effect of feed intake ($P < 0.001$)

**Figure 4.3** Density (Bmax) of beta-1 adrenergic receptors in kidney membranes of lambs raised in the cold or warm environment and fed at restricted or *ad libitum* level (values are means; N = 6. Different superscripts indicate significant difference, P < 0.05).

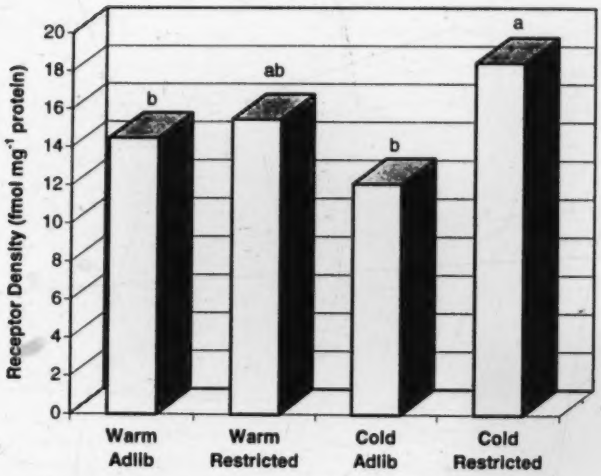



The table below shows the main effects of temperature and feed intake and their interaction (T*F) on density of beta-1 adrenoceptors in kidney

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Kidney | 32.43 | 36.22 | 30.29d | 38.36c | 1.78 | 0.07 |

Values are least squares means; N = 6
c, d indicate significant main effect of feed intake (P < 0.01)

**Figure 4.4** Density (Bmax) of beta-1 adrenergic receptors in liver membranes of lambs raised in the cold or warm environment and fed at restricted or *ad libitum* level (values are means; N = 6. Different superscripts indicate significant difference, P < 0.05).




The table below shows the main effects of temperature and feed intake and their interaction (T*F) on density of beta-1 adrenoceptors in liver

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| Liver | 14.98 | 15.28 | 13.29d | 16.96c | 0.99 | 0.06 |

Values are least squares means; N = 6
c, d indicate significant main effect of feed intake (P < 0.01)

# CHAPTER 5

## Beta-1 and Beta-2 mRNA Transcripts in Tissues of Sheep: Effects of Cold Exposure and Feed Restriction

### 5.1 INTRODUCTION

Beta-adrenergic receptors (β-adrenoceptors) belong to the superfamily of integral membrane proteins with seven transmembrane domains (Emorine et al. 1991; Jacobs 1994). The activation of β-adrenoceptors by catecholamines (epinephrine and norepinephrine) and their agonists leads to several metabolic effects such as stimulation of glycogenolysis in the muscles, increase in blood glucose levels, lipolysis, protein synthesis and a decrease in protein degradation (see Chapter One).

Pharmacological studies support the existence of three subtypes (β1, β2 and β3) of β-adrenoceptors in various tissues of animals. These three subtypes of β-adrenoceptors may co-exist in the same tissues (Galitzky et al. 1993) and in the same cells (Homburger et al. 1981; del-Monte et al. 1993). Beta-1 and β2-adrenoceptors were found to be present in skeletal muscles (Kim et al. 1992; Carlisle and Stock 1993), heart (Robberecht et al. 1983) and in kidney (Snavely et al. 1982), while the liver tissue was found to contain β2- and β3- adrenoceptors (Ruffolo 1994). In Chapters 3 and 4, radioligand binding studies indicated that β1- and β2- adrenoceptors are present in skeletal muscles, heart, kidney and liver tissues of sheep. However, these observations have not been confirmed at the level of the messenger ribonucleic acid (mRNA) for these important membrane receptors.

Attempts to ascertain the β-adrenoceptor subtypes present in animal tissues, using a number of β-adrenoceptor subtype specific agonists and antagonists in functional and radioligand binding studies, have not produced a clear delineation of the subtypes. This is because one agonist or antagonist can bind to two or more β-adrenoceptor subtype. However, the availability of the complementary deoxyribonucleic acid (cDNA) sequence data for some animals has allowed the development and utilization of molecular techniques, including Northern blots, nuclease protection assays, and reverse transcriptase-polymerase chain reaction techniques, to determine the presence and levels of tissue expression of β-adrenoceptor subtypes. Vivat et al. (1992) successfully used Northern blots to demonstrate the effects of progesterone on the expression of β2-adrenoceptors in pregnant rat myometrium. McNeel and Mersmann (1995) also used recombinant DNA techniques to identify the transcripts for the β-adrenoceptor subtypes, and to determine the relative expression of β1- and β2- adrenoceptor transcripts in different porcine adipose tissue depots.

Different sizes of transcripts for β- and β2- adrenoceptors have been reported for some tissues of some animals. The sizes of transcripts for the β1-adrenoceptors include 2.0 Kb in rat submaxillary gland (Bahouth et al. 1987), 2.4 Kb in human placenta, 2.5 Kb in rat muscle, heart, liver and kidney (Frielle et al. 1987), 2.6 Kb in human glial cells (Lopez-Barahona et al. 1996) and rat heart (Machida et al. 1990), 2.8 Kb in rat epididymal fat, 2.9 Kb in rat heart, 3.0 Kb in porcine heart (McNeel and Mersmann 1995), and 3.1 Kb in rat adipose and brain tissues (Scarpace et al. 1996). The sizes of transcripts for the β2-adrenoceptors include 1.8 Kb in porcine heart and 2.0 Kb in porcine adipose tissue and

lung (McNeel and Mersmann 1995). To date, there have been a few conclusive studies performed in ruminants regarding the DNA sequence and expression of β-adrenoceptors. Stoffel (1993) and Meyer et al. (1995) reported partial cDNA sequences for the bovine β2- and β3- adrenoceptor DNA, while studies by Tseng et al. (1995a) revealed β1-adrenoceptor mRNA species with a size of 2.6 Kb for heart, lung and brain tissues of fetal sheep.

In Chapters 3 and 4, pharmacological studies revealed that cold temperature exposure and feed restriction had significant influences on β1-adrenoceptor densities in skeletal muscles, heart, kidney and liver tissues of sheep. A number of studies have indicated that the β-adrenoceptor mRNA distribution in tissues correlate with that of the cognate receptors established by pharmacological studies (Arner et al. 1990; Rubio et al. 1995; Hadri et al. 1996; Ihl-Vahl et al. 1996; Scarpace et al. 1996). These parallel changes in the cellular densities of β-adrenoceptors and the steady state levels of their mRNAs suggest that receptor regulation is primarily at the transcriptional level, although other mechanisms such as stability of the mRNAs may be involved. Administration of glucocorticosteroids led to an increase in β-adrenoceptor density which is paralleled by an increase in β-adrenoceptor mRNA expression (Hadcock and Malbon 1988; Collins et al. 1988; Malbon and Hadcock 1988). This suggests that cellular activity could be regulated at the gene level by hormonal and environmental factors. It is, therefore, important to gain insights into the effects of cold exposure and feed restriction on the levels of β-adrenoceptor mRNAs.

In the present study, the expression of β1- and β2- adrenoceptor mRNAs in skeletal muscles, heart, kidney and liver tissues of sheep was investigated with the use of recombinant DNA techniques. The expression of β3-adrenoceptor mRNA was not studied because β3-adrenoceptor mRNA was not expected to be expressed in a major way in some of the tissues under study (see chapter one). In addition, the effects of cold exposure and feed restriction on the levels of β1-adrenoceptor mRNA in these tissues were determined.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Animals and Treatments

The animals and treatments are the same as described in Chapters 3 and 4. Briefly, twenty four Suffolk-cross, six-month-old wether lambs were slaughtered using captive bolt stunning and exsanguination after being raised for four months. These lambs, randomly divided into four treatment groups (WA, WR, CA and CR), were exposed to either warm (W; 23 ± 2 °C) or cold (C; 0 ± 2 °C) temperatures. They were fed experimental diets of alfalfa pellets (17 % crude protein, 9.08 MJ ME.$Kg^{-1}$DM; see Appendix 3 for feed composition) either *ad libitum* (A) or at a restricted level (R), on a daily basis. The lambs were initially adapted to treatments for 28 d before the experiment. The experiment was divided into three periods of five weeks each. In periods one and three, the treatments described above were imposed. During period two, WR and CR groups were removed from the experiment and these animals were fed *ad libitum* in a thermoneutral environment to prevent major declines in body condition prior to period three.

Samples of skeletal muscles (*biceps femoris*, *semitendinosus* and *gastrocnemius*), heart, kidney and liver tissues were collected immediately after slaughter, wrapped in aluminium foil and snap frozen in liquid nitrogen. The samples were stored at -72 °C for eleven months prior to RNA isolation and Northern blot analysis. McNeel and Mersmann (1995) reported that with respect to visual determination of the integrity of electrophoresed RNA and probing of Northern blot, the tissue may be stored for over a year, and the extracted RNA is also stable for over a year.

### 5.2.2 RNA Extraction

Total RNA was extracted from the samples by a method adapted from the single-step RNA isolation method of Chomczynski and Sacchi (1987). Briefly, about 1 g of each sample was finely ground in a pestle and mortar on dry ice. Frequent addition of liquid nitrogen kept the sample frozen during grinding. About 200 - 300 mg of the ground sample was homogenized with a Polytron homogenizer (Brinkmann Instruments, Switzerland) in 2.5 mL of TRIzol, a single phase reagent containing guanidinium salt and phenol (Gibco-BRL). The homogenized samples were incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. Chloroform (0.5 mL) was added to each tube. The tubes were securely capped and shaken vigorously by hand for 15 sec prior to incubation at room temperature for 3 min. The samples were centrifuged for 15 min at 12,000 x g at 4 °C, and the upper aqueous phase was transferred to a separate tube. Isopropyl alcohol (1.25 mL) was added to each tube. The mixture was vortexed briefly and incubated for 10 min at room temperature before centrifugation for

15 min at 12,000 x g at 4 °C. The supernatant was discarded and the RNA pellet was washed with 2.5 mL 75 % ethanol. The pellet was allowed to air-dry for 10 min before resuspension in 150 µL of RNase-free water. The resuspended RNA was quantified by spectroscopy measuring at $A_{260}$ (Gene Quant RNA/DNA Calculator, Pharmacia). The purity of the RNA was determined by comparing the ratio of absorbance at 260 nm to that at 280 nm. The ratio should be around 1.8; the actual ratios obtained were between 1.81 and 2.0 (nucleic acids have an optimum absorption at 260 nm while proteins have optimum absorption at 280 nm. The ratios of 1.8 and above indicate that more nucleic acids than proteins are present, while ratios above 2.0 indicate presence of DNA molecules). Aliquots of the total RNA were stored at -72 °C prior to electrophoresis.

### 5.2.3 Northern Blot Analysis

Total RNA (20 µg) was electrophoresed on denaturing 1 % agarose / 0.66 M formaldehyde gels and transferred to nitrocellulose membranes (Nitro-pure, Microns Separations Inc.) by overnight capillary diffusion (Sambrook et al. 1989). The success of the transfer was assessed by UV-light visualisation of ethidium bromide-stained RNA on both the gels and the membranes. The quality of the extracted total RNA was assessed by examining the relative intensities and integrity of the 28S and 18S ribosomal RNA bands. The positions of the 28S and 18S ribosomal RNAs were marked on the blotted membranes. The membranes were allowed to air-dry for 30 min before being baked in a vacuum oven (Fisher Scientific) for 2 h at 80 °C.

The blotted membranes were prehybridized in a solution containing 6 X SSPE [0.9 M NaCl, 60 mM $NaH_2PO_4$, 60 mM EDTA (ethylenediaminetetra-acetic acid) at pH 7.4], 0.5 % SDS (sodium dodecyl sulfate) and 5 X Denhardt's solution (0.1 % Ficoll 400, 0.1 % polyvinylpyrolidone, 0.1 % fraction V bovine albumin) for 2 h at 50 °C in an Auto Blot Hybridization incubator (Lab-Line Instruments). The prehybridization solution was removed and the membranes hybridized with fresh prehybridization solution containing denatured [$^{32}$P]-labeled cDNA probes. Hybridizations were carried out for 20 h at 50 °C. Following hybridization, the membranes were washed three times for 20 min at room temperature in 2 X SSPE containing 0.1 % SDS. The membranes were then washed for 20 min at 50 °C in 0.1 X SSC (15 mM NaCl, 1.5 mM trisodium citrate, pH 7.0) containing 0.1 % SDS. The membranes were sealed individually in plastic bags and exposed to Kodak X-Omat AR film with an intensifying screen in an X-ray cassette (Eastman Kodak, Rochester, New York) for 24 h at -72 °C. The exposed films were developed using Kodak X-Omat Processor (Health Sciences, Eastman Kodak, Rochester, New York).

### 5.2.4 Probes

The human β1-adrenoceptor cDNA probe (Frielle et al. 1987) and the human β2-adrenoceptor cDNA probe (Kobilka et al. 1987) cloned in *pBC12BI* vectors, were generously provided by Dr. R. J. Lefkowitz (Duke University Medical Center, Durham, North Carolina, USA). The recombinant plasmids were transformed into *E. coli* XL-1 Blue cells (Stratagene), amplified and isolated using the Plasmid Pure DNA Miniprep kit

(Sigma; Sambrook et al. 1989). The plasmids were digested with restriction enzymes to recover the plasmid DNA and cDNA inserts. The plasmids containing the human β1-adrenoceptor cDNA probe were digested with *Bam H1* (Gibco-BRL) and *Hind II* (Boehringer Mannheim), and the plasmids containing the human β2-adrenoceptor cDNA probe were digested with *Nco 1* and *Sal 1* (Gibco-BRL).

Following the restriction enzyme digestion, agarose gel electrophoresis was performed to isolate and recover the probes. The probes were labeled with [$\alpha$-$^{32}$P]-dATP (specific activity, 3,000 Ci mmol$^{-1}$; Amersham Life Sciences Inc.) using a random primer DNA labeling kit (Gibco-BRL; Feinberg and Vogelstein 1984). All labeled probes were used after 2 h of incubation at room temperature.

## 5.3 DATA ANALYSIS

In order to determine the sizes of the transcripts, total RNA samples and RNA molecular weight marker 1 (Boehringer Mannheim; Maniatis et al. 1982) were electrophoresed on denaturing 1 % agarose / 0.66 M formaldehyde gels and transferred to a membrane as described above. The sizes of the transcripts, 28S and 18S ribosomal RNAs were derived from a regression plot of logarithm of masses of RNA marker bands versus ratio of distance traveled by the marker to total length of gel. The relative abundance of transcripts was estimated by the absorbance intensity of bands measured on a scanning Imaging Densitometer (BIO-RAD, Richmond, CA, USA). The relative sample loading and concentration were determined by expressing all values on an equivalent 28S ribosomal RNA basis (de Leeuw et al. 1989; McNeel and Mersmann 1995; Rubio et al

1995). This was done by taking densitometric measurement of 28S ethidium bromide-stained band (in pictures of blots taken before hybridization) and using it to correct the densitometric value of the corresponding transcript.

The densitometric values were analysed as a 2 X 2 factorial using the general linear model procedures of SAS (SAS 1990). The model included a two-way analysis of variance and a Duncan multiple range test. The differences among means were compared using the Duncan multiple range test. The sources of variations were replicate, temperature and feed intake, and the interaction between them.

## 5.4 RESULTS

### 5.4.1 Gene Expressions of β1- and β2- Adrenoceptors

The electrophoresis patterns in all the blotted membranes were typical of intact biologically competent RNA with defined 28S and 18S ribosomal RNA bandings as demonstrated by the ethidium bromide staining of the agarose gels (Fig. 5.1). The sizes of the 28S and 18S ribosomal RNAs were derived from a regression plot of logarithm of masses of RNA marker bands versus the ratio of distance traveled by marker to total length of gel.

**Equation 5.1**: $\text{Log}(Y) = 1.695 - 2.404\,X.$

(0.06) (0.09)

Where; Y = size (mass) of RNA or transcript in Kb,

X = ratio of distances traveled as stated above.

The sizes of the 28S and 18S ribosomal RNAs were approximately 5.2 Kb and 2.0 Kb, respectively.

The human β1-adrenoceptor cDNA probe was used to determine tissue distribution of β1-adrenoceptor mRNA in skeletal muscles (*biceps femoris*, *semitendinosus* and *gastrocnemius*), heart, kidney and liver tissues of sheep. In all the tissues studied, Northern blotting indicated one predominant β1-adrenoceptor mRNA species with a size of about 2.2 Kb recognized by the human β1-adrenoceptor cDNA probe (Fig. 5.2 - 5.4). The size of these β1-adrenoceptor transcripts was derived from the above equation, relative to the positions of the 28S and 18S ribosomal RNAs. All the total RNA Northern blots demonstrated a good signal with low background, except the sections of the blots for samples of total RNA prepared from the kidney tissue which indicated a high background in the lanes.

In the case of β2-adrenoceptor mRNA distribution, the human β2-adrenoceptor cDNA probe did not indicate any detectable transcript for β2-adrenoceptor mRNA in all the tissues used for the investigation. Even at a lower stringency with hybridization and wash temperature of 45 °C, the human β2-adrenoceptor cDNA probe did not show β2-adrenoceptor mRNA transcript for any of the tissues.

### 5.4.2 Effects of Cold Exposure and Feed Restriction on Gene Expression

In order to determine the effects of cold temperature exposure and feed restriction on the distribution of the ovine β1-adrenoceptor mRNA, the relative abundance of the β1-

adrenoceptor mRNA transcripts was estimated by the absorbance intensity of bands measured on a scanning Imaging Densitometer (BIO-RAD, Richmond, CA, USA). The densitometric measurements are given in Table 5.1. These values were corrected for the densitometric measurements of 28S ribosomal RNA which was used as a sample loading control.

**5.4.2.1 Gene Expression of β1-Adrenoceptors in Skeletal Muscles**

For *biceps femoris*, both cold temperature exposure and feed restriction induced a pronounced increase in β1-adrenoceptor mRNA ($P < 0.001$), and their interaction was also significant, $P < 0.001$, (Table 5.1). The 2.2 Kb transcript of the β1-adrenoceptors was most intense in the cold-restricted group (CR). In the cold environment , the restricted group (CR) had more β1-adrenoceptor mRNA than the *ad libitum* group, CA ($P < 0.001$). A similar trend was observed in the warm environment where the restricted group (WR) had more β1-adrenoceptor mRNA than the *ad libitum* group, WA ($P < 0.01$). *The ad libitum* groups did not differ from each other when the two environments were compared ($P > 0.05$). However, the restricted group in the cold environment had a higher β1-adrenoceptor mRNA than its counter part in the warm environment ($P < 0.001$).

The results for *semitendinosus* followed a similar pattern as those for *biceps femoris* (Table 5.1). The main effects of temperature and feed intake and their interaction were significant ($P < 0.05$). The highest intensity and hence the most abundant β1-adrenoceptor mRNA transcript was observed in the CR group. In both cold and warm environments (CA : CR; WA : WR), feed restriction caused an increase in abundance of

β1-adrenoceptor mRNA transcripts ($P < 0.001$). The effect of cold temperature exposure was only significant ($P < 0.001$) with feed restriction, where cold temperature exposure caused an increase in β1-adrenoceptor mRNA.

In contrasts to the above, there was no treatment effect on the levels of β1-adrenoceptor mRNA in *gastrocnemius* muscle (Table 5.1). The interaction of temperature and feed intake did not have any effect either ($P > 0.05$). The densitometric values were similar for CA and CR ($P > 0.05$) in the cold environment, and also for WA and WR ($P > 0.05$) in the warm environment, indicating that feed restriction did not have any influence. Similarly, cold temperature exposure did not have any significant influence on the levels of β1-adrenoceptor mRNA for both *ad libitum* and restricted groups ($P > 0.05$).

#### 5.4.2.2 Gene Expression of β1-Adrenoceptors in Kidney, Heart and Liver

Statistical analysis revealed significant influences ($P < 0.001$) for both temperature and feed intake, and the interaction between them was also significant ($P < 0.001$) for β1-adrenoceptor mRNA in the case of the kidney (Table 5.1). β1-Adrenoceptor mRNA level was highest in the CR group. Feed restriction significantly increased β1-adrenoceptor mRNA in the cold environment ($P < 0.001$), but the increase in the warm environment was not significant ($P > 0.05$). Similarly, cold temperature exposure increased the β1-adrenoceptor mRNA level in the restricted group ($P < 0.001$), but did not have any influence in the *ad libitum* group ($P > 0.05$).

The influences of temperature and feed intake on the β1-adrenoceptor mRNA levels in the heart were in contrast to those observed for the skeletal muscles and the kidney (Table

5.1). The main effect of feed intake was significant ($P < 0.001$). Unlike feed restriction, cold temperature exposure did not have any significant effect on β1-adrenoceptor mRNA levels, and the interaction between temperature and feed intake was not significant ($P = 0.1$). The β1-adrenoceptor mRNA level was lowest in the CR group. Feed restriction reduced β1-adrenoceptor mRNA levels in the cold ($P < 0.001$) and in the warm ($P < 0.05$) environments. Comparison of the two environments showed that the β1-adrenoceptor mRNA levels were similar between WA and CA, and between WR and CR ($P > 0.05$).

The patterns of β1-adrenoceptor mRNA levels observed in the liver are similar to those for the skeletal muscles and the kidney (Table 5.1). There were significant effects of temperature ($P < 0.05$) and feed intake ($P < 0.01$). The effect of feed restriction was more pronounced than that of temperature. However, the interaction between temperature and and feed intake was not significant ($P > 0.05$). The CR group had the highest level of β1-adrenoceptor mRNA. In the cold environment, feed restriction increased the level of β1-adrenoceptor mRNA ($P < 0.01$). However, in the warm environment, the effect of feed restriction was not significant ($P > 0.05$), although WR had a higher level of β1-adrenoceptor mRNA than WA. The *ad libitum* groups had similar levels of β1-adrenoceptor mRNA ($P > 0.05$), while the restricted group in the cold environment had a higher level of β1-adrenoceptor mRNA than the restricted group in the warm environment ($P < 0.05$).

#### 5.4.2.3 β1-Adrenoceptor mRNA Distribution in Tissues of Sheep

In order to determine the relative abundance of β1-adrenoceptor mRNA in all the tissues, the densitometric values were normalized to that of the liver (value = 1) for each treatment group (Table 5.2). The liver had the lowest level of β1-adrenoceptor mRNA in each group. Except for CR, the heart tissue had the highest level of β1-adrenoceptor mRNA in each treatment group. *Biceps femoris* had the highest level of β1-adrenoceptor mRNA in CR group and was next to the heart in WR and CA groups. The kidney ranked number 3 in WR, CA and CR groups, and was fourth in WA group, in terms of β1-adrenoceptor mRNA level. The *semitendinosus* and *gastrocnemius* muscles ranked fourth or fifth in WR, CA and CR groups, respectively.

## 5.5 DISCUSSION

Catecholamines exert many of their physiological effects by interacting with beta-adrenergic receptors (β-adrenoceptors) on the cell surface of target cells. β-Adrenoceptors belong to a family of membrane proteins that mediate their effects through guanine nucleotide regulatory proteins, G-proteins (Emorine et al. 1991). The β-adrenoceptor subtypes, β1-, β2- and β3- adrenoceptors, have been distinguished based primarily on the ability of selected agonists and antagonists to stimulate or inhibit some physiological responses, or to bind to membrane preparations (Bylund et al. 1994). The development of recombinant DNA techniques has allowed the classification of β-adrenoceptor subtypes based on their genetic structures (Frielle et al. 1987; Kobilka et al. 1987; Machida et al.

1990). The present study examined the β1- and β2- adrenoceptor mRNA transcripts in some tissues of sheep.

### 5.5.1 Gene Expressions of β1- and β2- Adrenoceptors

Human β1-adrenoceptor cDNA probe was used to determine tissue distribution of β1-adrenoceptor mRNA in tissues of sheep. Northern blotting indicated one predominant β1-adrenoceptor mRNA species with a size of about 2.2 Kb recognized by the human β1-adrenoceptor cDNA probe (Fig. 5.2 - 5.4). The size of the transcripts was determined by using RNA marker 1, and in relation to the positions and sizes of 28S and 18S ribosomal RNAs, as described in the method. In the same way, the sizes of the 28S and 18S ribosomal RNAs were estimated to be 5.2 Kb and 2.0 Kb, respectively. These compare to the sizes of human and mouse 28S (5.1 Kb) and 18S (1.9 Kb) ribosomal RNAs (Darnell et al. 1986).

The presence of β1-adrenoceptor mRNA transcripts in ovine tissues indicates that β1-adrenoceptors are expressed in ovine tissues, and therefore, confirms the results of the radioligand binding assays, as reported in Chapters 3 and 4. Tseng et al. (1995a) had already reported the presence of β1-adrenoceptor mRNA transcripts with a size of 2.6 Kb for heart, lung and brain tissues of fetal sheep. Frielle et al. (1987) also used the human β1-adrenoceptor cDNA probe to identify β1-adrenoceptor mRNA transcripts (size, 2.5 Kb) in rat brain, heart, lung and liver. The ability of this heterologous DNA probe to detect β1-adrenoceptor mRNA transcripts in different animals suggests a certain degree of homology between the β1-adrenoceptors of these species. And indeed, Padbury et al.

(1995) reported that comparison of the predicted amino acid sequence of the β1-adrenoceptors from human, rat and sheep revealed overall amino acid identity of 85 - 90 % which approached 100 % within the transmembrane spanning regions.

Various sizes of transcripts of β1-adrenoceptor mRNA are reported in the literature for some tissues of animals. These sizes, some of which are similar to the 2.2 Kb obtained for ovine tissues in this study, range from 2.0 Kb to 3.1 Kb. These sizes include 2.0 Kb for rat submaxillary gland (Bahouth et al. 1987), 2.5 Kb for rat muscle, heart, liver and kidney (Frielle et al. 1987), 2.6 Kb for rat heart (Machida et al. 1990) and heart, lung and brain tissues of fetal sheep (Tseng et al. 1995a), 2.9 Kb for rat heart and 3.0 Kb for porcine heart (McNeel and Mersmann 1995). It is not clear whether these differences reflect any evolutionary or physiological significance. However, variation in sizes of transcripts is not a unique characteristic of β1-adrenoceptor mRNA. The mRNAs with different sizes have been reported for other receptors; for example, the size of mRNAs for the prolactin receptors varies considerably both between species and the organs studied (Kelly et al. 1991).

Hybridization of blotted membranes with human β2-adrenoceptor cDNA probe did not result in any detectable transcript for ovine β2-adrenoceptor mRNA. A similar result was obtained with a porcine β2-adrenoceptor DNA probe (donated by Dr. H. J. Mersmann, Baylor College of Medicine, Houston, Texas). However, these results do not indicate lack of β2-adrenoceptor mRNA in ovine tissues. Radioligand binding techniques indicated that β2-adrenoceptors are expressed in low concentrations in these tissues (Chapters 3 and 4). It is possible that β2-adrenoceptor mRNA is present in low

concentrations that could not be detected with the standard Northern blot analysis (Arner et al. 1990). It is also possible that the amount of total RNA (20 μg) used was not appropriate; increasing the amount of total RNA could have increased the chance of detecting the β2-adrenoceptor mRNA transcript. The detection of the transcript could be maximized by concentration of the mRNA, as occurs in the preparation of poly ($A^+$) RNA (McNeel and Mersmann 1995). The use of more sensitive methods, such as reverse transcriptase-polymerase chain reaction technique and ribonuclease protection assay, could have detected the β2-adrenoceptor mRNA transcript if it was present in low concentrations in these tissues. Ribonuclease protection assay can detect as little as 5 femtograms of target mRNA and it is at least ten times more sensitive than Northern blot analysis (Ambion Product Catalog 1995).

The negative data might be related to the conditions or the probe used for the transcript measurement. A change in hybridization temperature and / or use of other probes is / are also recommended (β1-adrenoceptor mRNA transcripts were not detected with porcine β1-adrenoceptor DNA probe, but were detected when human β1-adrenoceptor cDNA probe was used). The sequence of ovine β2-adrenoceptor mRNA is not yet known, and it is not possible to know whether the negative data relate to the homology of the human and sheep β2-adrenoceptor mRNAs. However, the sequence identity between the partial bovine β2-adrenoceptor cDNA (which is the closest to ovine) and the respective regions of the human, rat and mouse gene is 90 %, 85 % and 85 % (Meyer et al. 1995).

### 5.5.2 Effects of Cold Exposure and Feed Restriction on Gene Expression

The densitometric values of the β1-adrenoceptor mRNA transcripts (Table 5.1) were used to determine the effects of temperature and feed intake on the levels of β1-adrenoceptor mRNA in ovine tissues. The values were corrected for the densitometric values of 28S ribosomal RNA. De Leeuw et al. (1989) reported that 28S RNA is a more reliable internal control than β-actin because it appears to be a constant fraction of total RNA. This study was confirmed by McNeel and Mersmann (1995) and Rubio et al. (1995).

#### 5.5.2.1 Gene Expression of β1-Adrenoceptors in Skeletal Muscles

Cold temperature and feed restriction induced a pronounced increase in β1-adrenoceptor mRNA levels in ovine *biceps femoris* and *semitendinosus*. The interaction of the two also led to a significant increase in β1-adrenoceptor mRNA levels. It is possible that there is a causal relationship between cold temperature exposure or feed restriction and β1-adrenoceptor mRNA level in the muscle tissues. Cold exposure induced an increase in oxidative metabolism in skeletal muscles (Lefaucher et al. 1991), and densities of β-adrenoceptors correlate positively with oxidative capacity in skeletal muscles (Williams et al. 1984), observations which are consistent with a cold-induced increase in density of β1-adrenoceptors (Chapter 3). A number of reports indicate that an increase in β-adrenoceptor expression reflects corresponding increase in the steady state levels of β-adrenoceptor mRNAs (Arner et al. 1990; Hadri et al. 1996; Ihl-Vahl et al. 1996), suggesting that the effect of cold exposure could be at the level of the gene expression. In

this case, the cells may be regulating the density of β-adrenoceptors by controlling gene transcription, thereby having control on the levels of β-adrenoceptor mRNA.

In contrast to the above, temperature, feed intake and the interaction between them did not have any significant influence on β1-adrenoceptor mRNA level in *gastrocnemius* muscle. As discussed in Chapter 3, the three types of skeletal muscles used in this study are fast-twitch glycolytic or mixed fibre-types. However, differences in proportions of fibre-types could account for the differences in oxidative capacities and densities of β-adrenoceptors, and hence, differences in the levels of β-adrenoceptor mRNAs. Aalhus (1988) showed that the proportions of fibre-types are different in different skeletal muscles of sheep, and these differences could account for differences in β1-adrenoceptor mRNA levels in *biceps femoris*, *semitendinosus* and *gastrocnemius* muscles of sheep.

#### 5.5.2.2 Gene Expression of β1-Adrenoceptors in Kidney, Heart and Liver

The results indicate that feed restriction caused an increase in β1-adrenoceptor mRNA level in the kidney, in the cold and warm environments. However, the increase in the warm environment was not significant. Cold temperature exposure increased β1-adrenoceptor mRNA level in the kidney with feed restriction. Similar results were obtained for the liver tissue, except that the interaction between temperature and feed intake was not significant. In the case of the heart, the influences of cold temperature exposure and feed restriction on the levels of β1-adrenoceptor mRNA were in contrast to those observed for the kidney and the liver tissues. Feed restriction caused a decrease in β1-adrenoceptor mRNA levels in the heart. Unlike feed restriction, cold temperature

exposure did not have any significant effect on β1-adrenoceptor mRNA levels in the heart, and the interaction between temperature and feed intake did not lead to any significant effect.

Generally, it is not known how temperature or feed intake could affect gene expression in the tissues. Roth and Grunfeld (1985) reported that the structures and functions of receptors and hormones are influenced by the environment, including temperature. It is, therefore, possible for environmental factors to affect gene expression. Emorine et al. (1991) indicated that cellular activity is regulated at the gene level by hormonal and environmental factors. Such genetic regulation is often reflected by the presence, in the promoter regions of the genes, of conserved nucleotide sequences corresponding to binding sites for the various factors and modulators of transcription. Several putative glucocorticoid response elements (GRE), thyroid hormone response elements (TRE), as well as sites for other transcription factors have been identified in ovine β1-adrenoceptor gene promoter (Padbury et al. 1995). Direct or indirect effects of temperature and feed intake on these transcription factors would, therefore, have effects on transcription of β-adrenoceptor genes. Collins et al. (1988) reported that hormones such as catecholamines and glucocorticoids can alter transcription of β-adrenoceptor genes that would result in steady-state changes in β-adrenoceptor transcript levels. Glucocorticoids (Tseng et al. 1995b) and thyroid hormones (Lopez-Barahona et al. 1996) increased β1-adrenoceptor densities by activating β1-adrenoceptor gene transcription. In Chapter 2, I demonstrated that cold temperature exposure and feed restriction have effects on the concentrations of hormones in blood plasma. It is possible that the effects of cold temperature exposure and

feed restriction on β1-adrenoceptor mRNA levels are through alterations in hormonal concentrations. In chapter 2, results indicated that cold exposure increased plasma cortisol level, while feed restriction reduced plasma insulin level and increased plasma cortisol level. The increased plasma cortisol and reduced plasma insulin levels can increase the rate of β-adrenoceptor gene transcription, leading to increased β1-adrenoceptor mRNA levels as observed in the present study.

#### 5.5.2.3 β1-Adrenoceptor mRNA Distribution in Tissues of Sheep

The relative concentration of the β1-adrenoceptor mRNA varies across individual ovine tissues in each treatment group (Table 5.2). The heart tissue had the highest level of β1-adrenoceptor mRNA in three groups, and was second to the *biceps femoris* in CR group. The liver tissue had the lowest level of β1-adrenoceptor mRNA in all groups. The factors responsible for variations in β-adrenoceptor mRNA levels in different tissues are not well known. Studies by Arner et al. (1990) concluded that the variation in gene transcription may be a molecular mechanism underlying regional or tissue differences in β-adrenoceptor mRNA levels. Since these tissues perform different functions and are in different regions of the body, differences in types and number of transcription factors will lead to different rates of gene transcription.

Hormones are known to regulate gene transcription (Collins et al. 1988; Tseng et al. 1995b), and differences in relative expression of β1-adrenoceptor mRNA may be due to variations in the action of the hormones or other regulatory factors on β1-adrenoceptor gene transcription in different tissues. Post-transcriptional regulation such as

destabilization of β1-adrenoceptor mRNA could also result in regional differences in expression of β1-adrenoceptor mRNA. For example, catecholamines have post-transcriptional effects on β-adrenoceptor -transcript levels by promoting destabilization of β-adrenoceptor mRNAs (Hadcock et al. 1989). The mechanism of this destabilization of mRNA is not yet known. Phosphorylation of some factor involved in degradation of receptor mRNA may be activated, or the transcription and translation of such a factor may be induced. Collins et al. (1992) proposed that β-adrenoceptor mRNA destabilization may be cAMP-dependent, while Lohse (1993) proposed the involvement of sequences close to the poly (A)-tail of the mRNA. The mRNA decay rate (half-life) is a major determinant of mRNA abundance, and the half-lives of many mRNAs change in response to nutrient levels, cell growth rates, hormones and other factors (Ross 1996). Since these factors may have different levels of effects in different tissues, their influences could contribute to differences in mRNA levels among tissues, as observed in this study.

During tissue development, several factors and regulators of transcription interact to modulate the level of expression of various genes. Some factors may be cell and differentiation dependent and thus allow selective regulation of adrenergic sensitivity in accordance with cellular function (Emorine et al. 1991). As for other genes, several putative transcription regulatory sites for transcription factors and regulators such as TRE and GRE, have been identified in ovine β1-adrenoceptor gene (Padbury et al. 1995). The interaction of transcription factors at the transcription regulation sites will result in different transcription rates in different tissues. It follows that, cellular function determines the level of interaction of the transcription factors and modulators, which in

turn determine the level of gene expression. Since the functions of the tissues studied are different, one would have to expect differences in the levels of β1-adrenoceptor mRNAs, as observed in this study.

## 5.6 CONCLUSIONS

In the present study, Northern blotting analysis indicates one predominant species of β1-adrenoceptor mRNA transcript in ovine tissue with a size of 2.2 Kb. This compares to a size of 2.6 Kb for β1-adrenoceptor mRNA in the heart, lung and brain tissues of fetal sheep (Tseng et al. 1995a). These results support the expression of β1-adrenoceptors in tissues of sheep, as indicated by pharmacological studies in Chapters 3 and 4. Cold temperature exposure and feed restriction increased β1-adrenoceptor mRNA levels in *biceps femoris*, *semitendinosus*, kidney and liver tissues of sheep. In heart, feed restriction reduced β1-adrenoceptor mRNA levels. The effect of feed intake was more pronounced than that of temperature, and the interaction between them was significant when both effects were significant. In contrast to the above, cold temperature exposure and feed restriction did not have any effects on β1-adrenoceptor mRNA level in *gastrocnemius* muscle. The influences of cold temperature exposure and feed restriction on the levels of β1-adrenoceptor mRNA may be related to alterations in hormonal concentrations in blood plasma. The relative concentration of β1-adrenoceptor mRNA varies across individual ovine tissues studied. The possibility of positive and negative interactions among various transcription factors and modulators of β-adrenoceptor gene expression could lead to quantitative differences in the expression of β-adrenoceptor gene. Such

mechanisms could allow various specialized cells to independently modulate their adrenergic sensitivity in response to changes in hormonal and environmental conditions, leading to differences in β-adrenoceptors and β-adrenoceptor mRNAs as observed in this study.

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**Table 5.1**. Effects of temperature and feed intake on expression[z] of β1-adrenoceptor transcripts in tissues of sheep

| | Temperature | | Feed Intake | | SEM | T*F |
|---|---|---|---|---|---|---|
| | Warm | Cold | Adlib | Restrict | (Pooled) | (Prob.) |
| *Biceps femoris* | 2.2b | 4.1a | 1.7d | 4.7c | 0.22 | 0.0006 |
| *Semitendinosus* | 1.3b | 1.9a | 0.8d | 2.4c | 0.14 | 0.02 |
| *Gastrocnemius* | 1.5 | 1.4 | 1.5 | 1.4 | 0.27 | 0.6 |
| Kidney | 1.6b | 2.8a | 1.2d | 3.2c | 0.18 | 0.0005 |
| Heart | 9.5 | 9.3 | 13.2c | 5.6d | 0.81 | 0.1 |
| Liver | 0.3b | 0.5a | 0.2d | 0.5c | 0.06 | 0.3 |

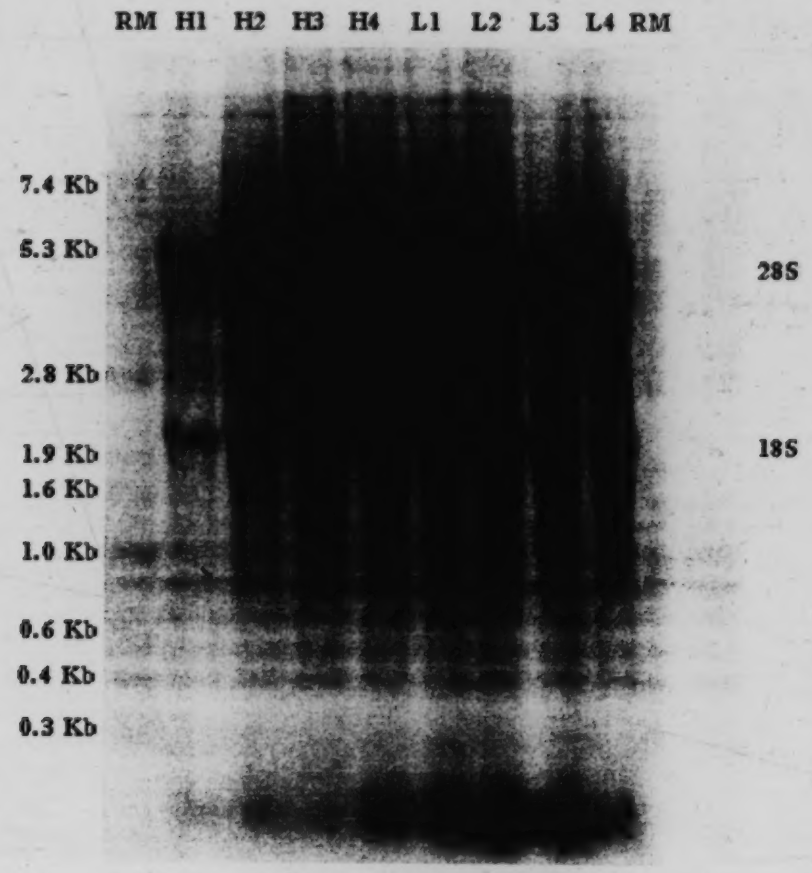
[z]Values are means, N = 4 (densitometric units in percent volume).
a, b indicate significant main effect of temperature ($P < 0.05$)
c, d indicate significant main effect of feed intake ($P < 0.001$)
T*F indicate probabilities for interaction of temperature and feed intake

Table 5.2. BAR-1 transcripts distribution[z] in tissues of sheep

| | WA | WR | CA | CR |
|---|---|---|---|---|
| Biceps femoris | 7.8 (3) | 7.9 (2) | 7.0 (2) | 9.8 (1) |
| Semitendinosus | 3.8 (5) | 4.8 (4) | 3.0 (5) | 4.6 (4) |
| Gastrocnemius | 9.2 (2) | 3.6 (5) | 4.9 (4) | 2.1 (5) |
| Kidney | 6.8 (4) | 5.2 (3) | 4.6 (3) | 6.8 (3) |
| Heart | 69.1 (1) | 17.3 (1) | 51.7 (1) | 7.0 (2) |
| Liver | 1 (6) | 1 (6) | 1 (6) | 1 (6) |

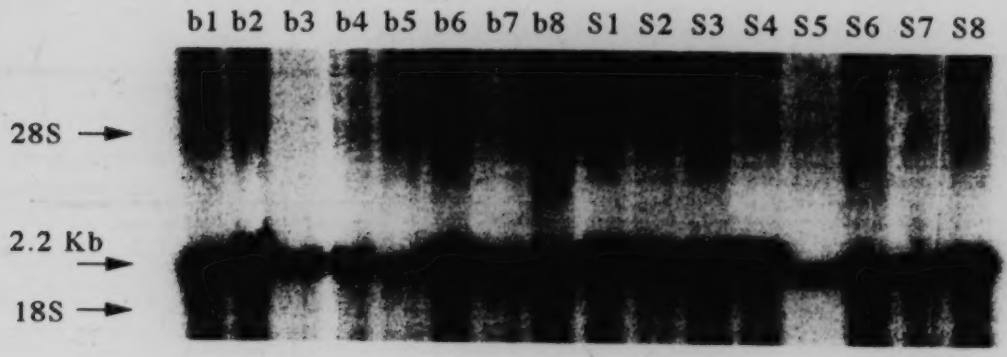
[z]Values were normalized to the densitometric value of the liver (value = 1) in each treatment group. Values in brackets are individual rankings, from highest to lowest values.

Figure 5.1 Electrophoresis patterns showing positions of 28S and 18S RNA bandings



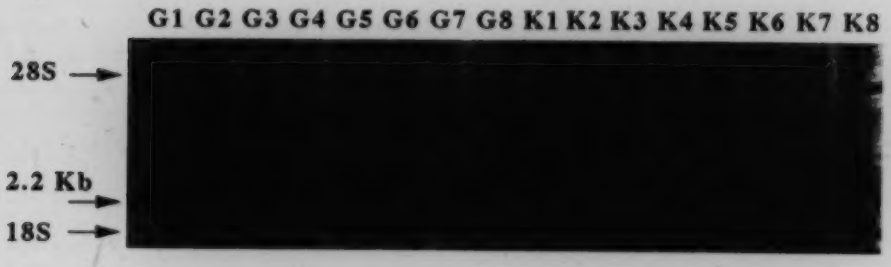

Total RNA samples (20 µg) of sheep heart (H) and liver (L) and RNA molecular weight marker 1 (RM) were electrophoresed as described under 'Materials and Methods'.The ordinate indicates migration of RNA molecular standards with weights in kilobase pairs. The sizes of 28S and 18S ribosomal were determined to be 5.2 Kb and 2.0 Kb, respectively.

**Figure 5.2** Beta-1 adrenoceptor mRNA transcripts in *biceps femoris* and *semitendinosus* of sheep



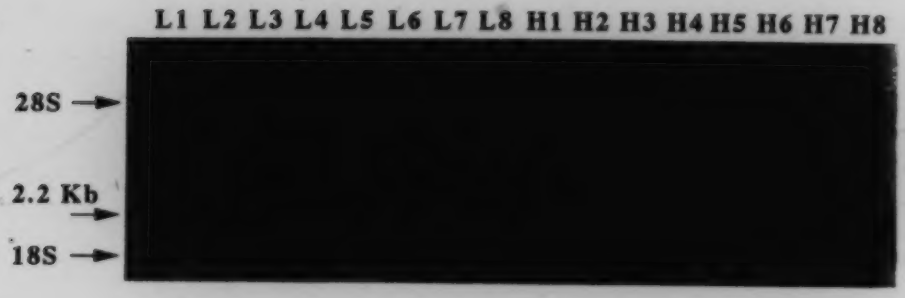

Northern blots performed with 20 µg of total RNA from *biceps femoris* (b) and *semitendinosus* (S) were hybridised with human beta-1 cDNA probe as described under 'Materials and Methods'. Samples were collected from sheep from four different treatment groups, warm *ad libitum* (1, 2), warm restricted (3, 4), cold *ad libitum* (5, 6) and cold restricted (7, 8). The size of beta-1 adrenoceptor mRNA transcript was 2.2 Kb.

**Figure 5.3** Beta-1 adrenoceptor mRNA transcripts in *gastrocnemius* muscle and kidney of sheep




Northern blots performed with 20 µg of total RNA from *gastrocnemius* muscle (G) and kidney (K) were hybridised with human beta-1 cDNA probe as described under 'Materials and Methods'. Samples were collected from sheep from four different treatment groups, warm *ad libitum* (1, 2), warm restricted (3, 4), cold *ad libitum* (5, 6) and cold restricted (7, 8). The size of beta-1 adrenoceptor mRNA transcript was 2.2 Kb.

**Figure 5.4** Beta-1 adrenoceptor mRNA transcripts in liver and heart tissues of sheep




Northern blots performed with 20 μg of total RNA from liver (L) and heart (H) tissues were hybridised with human beta-1 cDNA probe as described under 'Materials and Methods'. Samples were collected from sheep from four different treatment groups, warm *ad libitum* (1, 2), warm restricted (3, 4), cold *ad libitum* (5, 6) and cold restricted (7, 8). The size of beta-1 adrenoceptor mRNA transcript was 2.2 Kb.

# CHAPTER 6

## General Discussion and Conclusions

Catecholamines (epinephrine and norepinephrine) are major regulators of energy and protein metabolism. They initiate biological responses by first interacting with adrenergic receptors (adrenoceptors) on cell membranes. These receptors were originally classified as alpha- and beta- adrenoceptors on the basis of different responses to epinephrine, norepinephrine and synthetic adrenergic agents (Ahlquist 1948). Characteristically, all adrenoceptors have seven transmembrane domains, and they belong to a family of guanine nucleotide-regulatory protein (G-protein)-coupled receptors (Jacobs 1994). These receptors are found in nearly all peripheral tissues and on many neuronal populations within the central nervous system. This ubiquitous location of these catecholamine receptors presents a challenging area of studies which have been of major interest for many years. However, information concerning expression, concentration and activity of ovine beta-adrenoceptors is rare. The overall aim of this thesis was to investigate the expression of beta-adrenoceptors in ovine tissues and to investigate how cold temperature exposure and feed restriction affect the expression of these receptors.

### 6.1 Expression of Beta-Adrenergic Receptors in Ovine Tissues

Functional studies, radioligand binding studies and recombinant DNA techniques have been used to demonstrate the expression of all three subtypes of β-adrenoceptors (β1, β2

and β3). In this study, radioligand binding technique, using [$^3$H]dihydroalprenolol as a radioligand, and a recombinant DNA technique were used to demonstrate the expressions of β1- and β2- adrenoceptors in skeletal muscles, heart, kidney and liver tissues of sheep. β3-Adrenoceptors have been demonstrated primarily in adipose tissue (Emorine et al. 1991) and were not expected to be expressed in a major way in the tissues under study. On the other hand, data with other species suggested that β1- and β2- adrenoceptors would be the dominant subtypes (see chapter one). It was, therefore, decided to focus on the study of β1- and β2- adrenoceptors. The study of β3-adrenoceptors would be of interest with respect to adipose tissue.

Evidence supports the existence of β1- and β2- adrenoceptor subtypes in skeletal muscles of animals. Thorin et al. (1986) reported that the adrenoceptor mediating thermogenesis in skeletal muscles is the β1 subtype. However, this has not been supported by the work of Fagher et al. (1986, 1988) which showed a major thermogenic role for β2-subtype in skeletal muscles. Functional role of β2-adrenoceptors in muscle protein accretion and thermogenesis also supports the presence of β2-adrenoceptors in skeletal muscles (MacRae et al. 1988, Astrup et al. 1989, Choo et al. 1992). In chapter 3, radioligand binding studies indicate that β1- and β2- adrenoceptors coexist in *biceps femoris*, *semitendinosus* and *gastrocnemius* muscles of sheep. The binding studies also indicate that there was no negative co-operativity or the presence of multiple binding sites for binding of dihydroalprenolol (DHA). This result is similar to those of Reddy and Engel (1979), Reddy et al. (1979) and Kim et al. (1992), who reported that DHA binding to rat skeletal muscle membranes was saturable and with a single class of receptor sites.

This was despite the fact that DHA binds to both β1- and β2- adrenoceptors, thus indicating that DHA has similar affinities for both subtypes.

In all the three skeletal muscles studied, β1-adrenoceptor density was more than that of the β2-adrenoceptors, with β1-adrenoceptors being about 56 % to 87 % of the total. This result is similar to that obtained for rat *soleus* muscles (Kim et al. 1992). However, Elfellah and Reid (1987) concluded that β-adrenoceptors in *gastrocnemius* and *soleus* muscles of pigs are predominantly, if not exclusively, of the β2-subtype. This is supported by evidence that *soleus* muscles of cat (Minneman et al. 1979 a), bovine *musculus trapezium* (Ijerman et al. 1984) and *plantaris* muscles of rat (Kim et al. 1992) were found to have β2-subtype only.

The present results also show that densities of β1-adrenoceptors in *biceps femoris* of the sheep are different from those of *semitendinosus* and *gastrocnemius* muscles, with the *biceps femoris* having more β1-adrenoceptors than either *semitendinosus* or *gastrocnemius* muscle. There were no differences obtained for densities of β2-adrenoceptors for the three types of skeletal muscles. The three skeletal muscles are mixed fibre-types, but this can not explain the differences in densities of β1-adrenoceptors. The proportions of the different fibre-types are different in these muscles. This variation in fibre types accounts for the variation in oxidative capacity of skeletal muscle, and density of β-adrenoceptors was found to correlate with the oxidative capacity of the skeletal muscles (Williams et al. 1984). Therefore, the variation in densities of β1-adrenoceptors may be related to the variation in the proportions of fibre-types in these three types of skeletal muscles (Aalhus 1988, chapter 3).

Physiological studies indicate that β1- and β2- adrenoceptors are responsible for the chronotropic control of sino-atrial node in cat heart (Carlsson et al. 1977). Hedberg et al. (1980) reported an apparent homogenous population of β1-adrenoceptors in the left ventricle of cat and guinea pig, whereas the atria of both species contained both β1- and β2- adrenoceptors in the ratio of approximately 3:1. In chapter 4, the results indicate the presence of β1- and β2- adrenoceptors in ovine heart, in the ratio of approximately 8:1. Other reports indicate the ratio of 4:1 in rat heart (Minneman et al. 1979 b), 3:1 in human heart (Bjornerheim et al. 1991) and 1:1 in human auricles (Robberecht et al. 1983). The above reports indicate variations due to tissue type, physiological conditions and species. These differences could also be due to sex, age and nutritional states of the animals used for the different studies. The presence of β1-adrenoceptors in ovine heart was first demonstrated by Tseng et al. (1995 a), who worked on fetal sheep, but this study was not designed to assess the relative densities of β1- and β2- adrenoceptors.

Beta-adrenoceptors regulate renal blood flow, renin release and a number of other functions in mammalian kidney (Insel and Snavely 1981). In chapter 4, the results indicate that ovine kidney contains both β1- and β2- adrenoceptors, in the ratio of about 6:1. This result is similar to other reports which also indicate the coexistence of β1- and β2- adrenoceptors in the kidney. However, a number of reports indicate variation in densities of β1- and β2- adrenoceptors. Snavely et al. (1982) indicated the presence of β1- and β2- adrenoceptors in rat renal cortex in the ratio of 7:3. In other reports by Snavely et al., the ratios were 3:2 (1985 a) and 3:1 (1985 b) in rat renal cortex. This variation in concentrations of β1- and β2- adrenoceptors in the kidney may be related to

the differential regulation of the receptors and the level of catecholamines in blood plasma (Snavely et al. 1985 b). Species difference could also account for the variation in densities of β-adrenoceptors in kidneys of different animals. The results also indicate that DHA binding to ovine kidney membranes showed a single class of binding sites. Similar results were obtained for skeletal muscles (chapter 3) and rat kidney (Snavely et al. 1982). This is in contrast to reports by Woodcock and Johnston (1980 a, b) which indicated the presence of multiple classes of binding site, with negative co-operativity, in rat kidney membranes.

In the liver, catecholamines regulate a variety of functions, including key steps in carbohydrate, lipid and amino acid metabolism (Hadley 1984, Cruise et al. 1985). The present study (chapter 4) indicates that β1- and β2- adrenoceptors are present in ovine liver, in the ratio of approximately 3:1. The expression of β1-adrenoceptors in liver is supported by the work of Frielle et al. (1987). Ishac et al. (1992) showed that β2-adrenoceptors are expressed in the liver. The low concentration of β2-adrenoceptors in the liver, obtained in this study, is similar to that of rat liver (Ishac et al. 1992). In contrast to the above, Wolfe et al. (1976) and Kawai and Arinze (1983) reported a much higher concentration of β-adrenoceptors in rat liver.

Attempts to ascertain the β-adrenoceptor subtypes present in animal tissues, using a number of specific agonists and antagonists in functional and radioligand binding studies, have not produced a clear delineation of the subtypes. This is partly because, at high doses, specific agonists and antagonists can also bind to other receptors (Bylund et al. 1994). However, the development of molecular cloning techniques has provided new

tools to address this problem. In chapter 5, human β1- and β2- cDNA probes were used to determine the distribution of β1- and β2- adrenoceptor mRNAs, respectively, in tissues of sheep. Northern blot analysis indicated one predominant β1-mRNA species with a size of about 2.2 Kb in all the tissues studied. But hybridization with the human β2-cDNA probe did not detect any β2-mRNA transcript.

The presence of β1-mRNA transcripts in ovine tissues indicates that β1-adrenoceptors are expressed in ovine tissues. This, therefore, confirms the results of the radioligand binding studies in chapters 3 and 4. Tseng et al. (1995 a) reported the presence of β1-mRNA transcripts with a size of 2.6 Kb for heart, lung and brain tissues of fetal sheep, a result which is similar to that of the present study. The probe used for this study was used by Frielle et al. (1987) to detect β1-mRNA transcripts with a size of 2.5 Kb in rat muscle, heart, liver and kidney. This suggests that there is a certain degree of homology between the β1-adrenoceptors of rat, sheep and human. This suggestion is supported by the finding that there is overall amino acid identity of 85 to 90 % which approached 100 % within the transmembrane spanning regions of the β1-adrenoceptors of these species (Padbury et al. 1995).

In the case of β2-mRNA, the inability of the human β2-cDNA and porcine β2-DNA probes to detect β2-mRNA transcript does not necessarily mean that there is lack of β2-adrenoceptors in ovine tissues. Low concentrations of β2-adrenoceptors were determined using radioligand binding assays in chapters 3 and 4. It is possible that β2-mRNA is present in low concentrations that could not be detected with the standard Northern blot analysis (Arner et al. 1990). It is possible that the amount of total RNA (20 μg) used was

not appropriate; increasing the amount of total RNA would have increased the chance of detecting the β2-adrenoceptor transcripts. The detection of these transcripts could be maximized by concentration of the mRNA, as occurs in the preparation of poly ($A^+$) RNA (McNeel and Mersmann 1995). The use of more sensitive methods, such as reverse transcriptase-polymerase chain reaction technique and ribonuclease protection assay, could have detected the transcripts if they were present in low quantities in these tissues. Ribonuclease protection assay can detect as little as 5 femtograms of target mRNA and it is at least ten times more sensitive than Northern blot analysis (Ambion Product Catalog 1995) The negative data might be related to the conditions or the probes used for transcript measurement. A change in hybridization temperature or use of other probes is recommended. The sequence of ovine β2-adrenoceptor transcript is not yet known, and it is not possible to know whether the negative data relate to the homology of the human or porcine and ovine β2-adrenoceptor transcripts. However, the sequence identity between the partial bovine β2-adrenoceptor cDNA (the closest to ovine) and the respective regions of the human, rat and mouse gene is 91 %, 85 % and 85 % (Meyer et al. 1995).

## 6.2 Effects of Cold Exposure and Feed Restriction on Expression of Beta-Adrenoceptors

Radioligand binding and molecular techniques have demonstrated alterations in the β-adrenoceptor binding sites in a number of pharmacological, physiological and pathological states. Bao (1990) reported that the number of myocardial β-adrenoceptors increased while their affinity decreased progressively following endotoxin injection in

rats. The density of β-adrenoceptors decreased in hypertensive rats (Watanabe et al. 1991), but increased in adrenalectomized rats (Kawai and Arinze 1983). In chapters 3, 4 and 5, it is shown that cold exposure and feed restriction have effects on expression of β-adrenoceptors.

The results indicate that cold exposure and feed restriction increased the densities of β1-adrenoceptors in skeletal muscles (*biceps femoris*, *semitendinosus* and *gastrocnemius*), kidney and liver tissues of sheep. In the heart, the influence of feed restriction was different, with the feed restriction causing reduced density of β1-adrenoceptors. The variation in densities of β1-adrenoceptors in ovine tissues may be due to genetic make-up and the activities of the tissues. In the case of the heart, a large heart requires a larger force of contraction (Guyton 1976), and a greater number of β-adrenoceptors is required to produce a large force of contraction. In chapter 4, results showed that animals in feed restricted groups had smaller hearts than those in *ad libitum* groups, and these animals are expected to have less β1-adrenoceptors, as observed in this study. On the other hand, the increased β1-adrenoceptors by feed restriction in other tissues could increase such functions as glycogenolysis, glycolysis, and amino acid and lipid metabolism. Such mechanisms result in mobilization of substrates in support of energy metabolism. Therefore, variation in effects of temperature and feed intake on these tissues could be due to their different demands. The reduction in β1-adrenoceptors in relation to reduced size of the heart may be more important in the heart, while the increased β1-adrenoceptors may be important for other tissues to support energy metabolism.

Neither cold exposure nor feed restriction had effect on density of β2-adrenoceptors. Cold temperature and feed restriction reduced the receptor binding affinities in kidney and liver, and a high concentration of catecholamines would, therefore, be needed to effect responses. Since the concentrations of endogenous catecholamines remain high in a cold environment (Sasaki and Weekes 1984) and during feed restriction, the change observed in receptor density is likely more important than that of the change in binding affinity. Since the effectiveness of hormones is a function of their concentrations, binding affinities and their receptor densities (Roth and Grunfeld 1985), these results imply that cold exposure and feed restriction could potentially reduce (in heart) and increase (in other tissues) the metabolic responsiveness of tissues to catecholamines. The degree of physiological changes expected as a result of feed restriction will be significantly higher than the changes expected as a result of cold exposure. These inferences will be different in cases where the physiological responsiveness do not correlate with the binding data and when statistical significance does not necessarily mean physiological significance. For example, when spare receptors are involved, an occupancy of a fraction of the receptors could lead to production of a submaximal concentration of cAMP, which results in a maximal physiological response (Roth and Grunfeld 1985). The existence of spare receptors would, therefore, have unpredictable consequences for the comparison of binding data and physiological data for β-adrenoceptors.

In *gastrocnemius* muscle, neither cold exposure nor feed restriction had influences on β1-mRNA levels. In other tissues studied, the influences of cold exposure and feed

restriction on β1-mRNA levels parallel those of β1-adrenoceptor densities. This suggests that the effects of cold exposure and feed restriction may be at the level of gene transcription (see discussion below). A number of studies indicate that changes in β-adrenoceptor densities reflect corresponding changes in the levels of β-adrenoceptor mRNAs (Arner et al. 1990, Hadri et al. 1996, Ihl-Vahl et al. 1996). These parallel changes in the cellular densities of β-adrenoceptors and the steady-state levels of their mRNAs indicate that the receptor regulation is primarily at the gene level. The observed changes could be due to changes in the rates of transcription, stability of the mRNAs, or both. The lack of correlation between β1-adrenoceptor density and its mRNA level in *gastrocnemius* muscle is similar to reports by Ishac et al. (1992), Reynisdottir et al. (1993) and MacEwan and Milligan (1996). This suggests a complex regulation, which involves translational and / or post-translational regulation. In this case, changes in receptor number could be effected by changes in translational efficiency of existing mRNA, stabilization of the receptors from proteolytic degradation, downregulation or recycling of sequestered (internalized) receptors (Collins et al. 1992, Caron and Lefkowitz 1993, Marullo et al. 1995).

Table 6.1 shows an index of transcriptional and post-transcriptional contributions for each tissue in each treatment group. The values were derived by comparing the β1-adrenoceptor densities and corresponding β1-adrenoceptor mRNA densities. Tissues with high receptor levels (eg. Heart and *biceps femoris*) had lower transcriptional - post-transcriptional indices than those with low receptor levels (eg. liver). A low index could be due to a high rate of transcription (high mRNA level) or slower mobilization and

translation of mRNAs (low receptor level). On the other hand, a high index could be due to a low rate of transcription (low mRNA level) or faster mobilization and translation of mRNAs (high receptor level) (Darnell 1986). The influences of cold exposure and feed restriction on the transcriptional - post-transcriptional indices were opposite to those of β1-adrenoceptor densities and β1-adrenoceptor mRNAs. The indices are high where the receptor densities are low.

Generally it is not known how temperature or feed intake affect gene expression in tissues. Emorine et al. (1991) reported that cellular activity is regulated at the gene level by hormonal and environmental factors. Such genetic regulation is often reflected by the presence of sites, in the promoter regions of the genes, for the various transcription factors and modulators. Padbury et al. (1995) have identified several putative glucocorticoid and thyroid response elements, as well as sites for other transcription regulatory factors in ovine β-adrenoceptor gene promoter. Sites for general transcription factors, including Sp1 and AP2, which are important in regulation of core promoter activity of several genes, were also identified. The interaction of hormones or other transcription modulation factors with these sites would result in regulation of gene transcription. Direct or indirect effects of temperature or feed intake on these transcription factors would, therefore, have effects on transcription of genes. Catecholamines, glucocorticoids and thyroid hormones can alter transcription of β-adrenoceptor genes that would result in steady-state changes in β-adrenoceptor transcript levels (Collins et al. 1988, Tseng et al. 1995 b, Lopez-Barahona et al. 1996). In chapter 2, it is demonstrated that cold exposure and feed restriction increased plasma cortisol level and feed restriction

reduced plasma insulin and thyroid hormone (triiodothyronine, T3) levels. Reports also indicate that cortisol upregulates (Reynisdottir et al. 1993), insulin downregulates (Kang et al. 1993) and thyroid hormones upregulate or downregulate (Liggett et al.1989) β-adrenoceptors. The changes in β-adrenoceptor number due to the effects of these hormones could be as low as 2-fold or as high as 50-fold. The increased plasma cortisol level and the reduced plasma levels of insulin and T3 can increase the rate of β-adrenoceptor gene transcription, leading to high levels of β1-adrenoceptor mRNA, and hence, high level of β1-adrenoceptors, as observed in this study. It is, therefore, possible that the effects of cold exposure and feed restriction on β1-mRNA levels are through the alterations in hormonal concentrations in blood plasma.

Post-transcriptional regulation such as destabilization of β1-mRNA could also result in differences in expression of β1-adrenoceptors. The mechanism of destabilization of mRNA is not yet known. Phosphorylation of some factor involved in degradation of receptor mRNA may be activated, or the transcription and translation of such a factor may be induced. Collins et al. (1992) proposed that β-adrenoceptor mRNA destabilization may be cAMP-dependent, while Lohse (1993) proposed the involvement of sequences close to the poly ($A^+$)-tail of the mRNA. The mRNA decay rate (half-life) is a major determinant of mRNA abundance, and the half-lives of many mRNAs change in response to nutrient levels, cell growth rates, hormones and other factors (Ross 1996). Since these factors may have different levels of effects in different tissues, their influences could contribute to differences in mRNA levels among tissues, as observed in study.

Hadcock et al. (1989) showed that catecholamines promote destabilization of β-adrenoceptor mRNAs. Christopherson et al. (1978) showed that cold exposure increased plasma catecholamine (epinephrine and norepinephrine) levels. This could mean decrease in β1-mRNA and β1-adrenoceptor levels in the cold, as observed in the ovine heart (CR group) in this study. This explanation is not consistent with the results for other tissues studied, where cold exposure caused increased β1-adrenoceptor densities. In these tissues, the increased cortisol level and the decreased insulin and T3 levels may be more important than the increased level of catecholamines. Taken together, the variation in the distribution of β-adrenoceptors and β-adrenoceptor mRNAs in different tissues may be due to variations in the action of the hormones or other regulatory factors in these tissues. It is also possible for various transcription modulators to interact differently in different tissues, leading to variations in both mRNA and β-adrenoceptor levels.

Within the treatment groups, there is variation in gene expression, as demonstrated by the high coefficient of variation (see Tables A2.1 and A2.2 in Appendix 2). Candelore et al. (1996) described a naturally occurring mutation in the human adipose tissue β3-adrenoceptor gene which resulted in substitution of tryptophan residue at position 64 in the first intracellular loop with an arginine residue. Green et al. (1993) identified several naturally occurring variants of the human β2-adrenoceptors. One of these polymorphisms is a mutation occurring in the fourth transmembrane spanning domain with isoleucine substituted for threonine at amino acid-164 within the proposed ligand binding pocket. This mutation resulted in a 2- to 4- fold decrease in ligand binding, coupling to $G_s$-proteins and receptor sequestration. The reduced receptor sequestration could increase

binding data, leading to variation in receptor number. The presence of beta-adrenoceptor variants may explain, in part, the variability in gene expression and responses to catecholamines, as noted by Green et al. (1993) and Candelore et al. (1996). It is possible that variation in gene expression could relate to the variability in efficiency of growth and metabolic rate (see Table A2.3 in Appendix 2) in ruminants. However, polymorphism has not been demonstrated for beta-adrenoceptors in ruminants.

The present study indicated that the sheep had much greater β1- than β2-adrenoceptors in skeletal muscles, heart, kidney and liver tissues. There is no information in the literature on the relative densities of β1- and β2- adrenoceptors in tissues of sheep. Using [$^3$H]DHA as a radioligand, Watt et al. (1991) determined densities of 28 to 52 fmol.mg$^{-1}$ protein for β-adrenoceptors in sheep adipose tissue, which are similar to values obtained for skeletal muscles and kidney in chapters 3 and 4. Tseng et al. (1995) also used [$^3$H]DHA to determine densities of 164 to 209 fmol.mg$^{-1}$ protein for β-adrenoceptors in sheep heart, and these values are similar to values obtained for sheep heart in the present study. However, the studies of Watt et al. (1991) and Tseng et al. (1995) did not assess the relative concentrations of β1- and β2- adrenoceptors in these tissues.

With exception of the heart and kidney where more β1-adrenoceptors are expected, the skeletal muscles and the liver were expected to have more β2-adrenoceptors, as most studies on other animals seem to indicate (see chapters 1, 3 and 4). In chapters 3 and 4, the densities of β2-adrenoceptors were obtained by calculating the difference between the total and β1-adrenoceptors obtained in the absence and presence of terbutaline,

respectively. Terbutaline has a high affinity for β2-adrenoceptors, but the affinity for β1-adrenoceptors is about 1,000 times less (Bylund et al. 1994). In this study, 5 μM of terbutaline was used to saturate β2-adrenoceptors, but was low enough not to affect β1-adrenoceptors. However, since the use of terbutaline was not tested with sheep tissues, it is not known whether the concentration of terbutaline chosen was enough to saturate all the binding sites for β2-adrenoceptors. If that was not the case, this could complicate the binding data. However, Northern blot analysis did not detect any transcripts for β2-adrenoceptors in these tissues, with human cDNA and porcine DNA probes, even at a lower stringency with hybridization and wash temperature of 45 °C. This seems to indicate that the transcripts are present in low concentrations (use of poly ($A^+$) RNA or a more sensitive ribonuclease protection assay can prove this), which could result in low concentrations of β2-adrenoceptors as observed in this study.

The variation in relative densities of β1- and β2-adrenoceptors in tissues of different animals could be due to species differences, diets, age and nutritional states of the animals used for the different studies. The variation could also be due to different media compositions and assay conditions used (Mersmann and McNeel 1992). Differential transcription is probably the most frequent basis of differential protein synthesis, and a cell might achieve differential protein synthesis by transcriptional control, translational control, or both (Watson et al. 1992). It makes sense for a cell not to make more molecules than it needs, and there is no cell in which all genes are controlled in the same manner (Darnell 1986). As discussed above, the possibility of negative and positive interactions among various modulators (eg. Hormones) of β-adrenoceptor gene

expression could lead to quantitative differences in the expression of each β-adrenoceptor gene.

The three β-adrenoceptor subtypes have distinct genetic regulatory properties, thus allowing for modulation of adrenergic responsiveness of specific cells implicated in a particular physiological function. For example, the β2-adrenoceptors contain two, β1-adrenoceptors one, and β3-adrenoceptors no PKA-phosphorylation site (Lohse 1993). Correspondingly, there is pronounced PKA-mediated downregulation of the β2-adrenoceptors, much less for the β1-adrenoceptors and none for the β3-adrenoceptors. Such conditions could lead to more β1-adrenoceptors than β2-adrenoceptors in the plasma membranes as observed in this study. In a study of co-ordinated expression of β-adrenoceptor genes during the adipose conversion of 3T3-F442A cells, Emorine et al. (1991) found that these cells expressed much more β1-adrenoceptors than β2-adrenoceptors at the diffrentiated stage, but the levels of β2-adrenoceptors increased in mature adipocytes. This shows that the stage of development of a cell can affect the differential expression of the β-adrenoceptor subtypes.

The physiological relevance of this variable β-adrenoceptor regulation is not known. Epinephrine and norepinephrine have similar affinities for β1-adrenoceptors (Ruffolo 1994). This is an advantage to the sheep since each tissue has predominantly β1-adrenoceptors in this study. In the case of β2-adrenoceptors, epinephrine has a greater affinity than norepinephrine (Ruffolo 1994), and a higher concentration of norepinephrine will be needed to have a similar effect as epinephrine. In the cold environment, the sympathetic nervous system is stimulated (Graham et al. 1981) and more norepinephrine

than epinephrine will be released. In addition to the increasing concentration of norepinephrine, it would be an advantage to the animals if their tissues have predominantly β1-adrenoceptors, as observed in this study. Interestingly, the binding data fit to a one-site model indicating the presence of one class of binding sites. It may not make any difference whether it is β1- or β2- adrenoceptor, since the binding characteristics and the signal transduction pathways are similar for both receptor subtypes.

### 6.3 About the Research: Limitations and Justifications

The methods used in isolating crude plasma membranes were originally used to isolate plasma membranes from skeletal muscles of rat, rabbit and human (Ohlendieck et al. 1991). These methods were applied to sheep tissues with the assumption that the methods will not alter the results of the experiments. This assumption may be wrong because variation between studies from different laboratories might be a result of the assay conditions or membrane preparation methods used, as indicated by Mersmann and McNeel (1992). Methods that work well for one species of animals may not work the same way for another species of animals (Mersmann 1995). Similarly, a method may be applicable to a particular tissue, not to the other, because of possible variations in structures of the constituents of these tissues. Preliminary study and the radioligand binding study indicated that the methods were suitable for isolation of plasma membranes from sheep tissues. However, it is not known whether the methods did contribute to the variations in the binding data obtained in this study.

The phosphate or Tris buffer was used to maintain the pH of the solution at a desirable range. It is important to maintain a relatively high buffering capacity because of addition of hydrochloride salts of catecholamines or ligands to the incubation medium (Williams and Lefkowitz 1978). $MgCl_2$ can be added to the incubation medium to increase specific binding (Williams et al. 1978), although at a concentration above 10 mM, it can reduce specific binding (Mersmann and McNeel 1992). $MgCl_2$ (1 mM) was added to buffer A, but not buffer B, why this was done is not known. KCl was added to the buffer to dissolve the muscle filaments, it can also be added to normalize the binding data and to provide isotonicity (NaCl can also perform these functions, as indicated by Mersmann and McNeel 1992).

Other components may be added to the buffer in order to preserve the integrity of some membrane components. Williams and Lefkowitz (1978) reported that a variety of combinations of protease inhibitors should be added to the lysing and incubation media. Aprotinin, leupeptin, pepstatin, benzamidine, iodoacetamide and phenylmethylsulfonyl fluoride (PMSF) were used as protease inhibitors in this study. By preventing proteolytic degradation of the receptors, the protease inhibitors help to normalize the binding data as well as increasing the specific binding. The EDTA, included in the buffer, is a universal complexing agent for most divalent cations. It is used to inactivate modifying enzymes which are preferentially active in the presence of divalent cations, eg. $Mg^{++}$ and $Ca^{++}$. It inhibits metallo-proteases, thus preventing proteolytic degradation of the receptors.

A radioligand used in a binding study must be biologically active, thus demonstrating that it has an observable effect on the physiologically active receptors. Tritiated

dihydroalprenolol ([$^3$H]DHA) used for this study is a potent antagonist of isoproterenol-stimulated adenylate cyclase in frog erythrocyte membrane (Williams and Lefkowitz 1978). It has a very high affinity for β1- and β2- adrenoceptors, but it does not bind to β3-adrenoceptors (Bylund et al. 1994). [$^3$H]DHA has been used as a radioligand for binding studies involving a wide variety of tissues from rat, turkey, frog, dog, cattle and human (Williams and Lefkowitz 1978). Tseng et al. (1995a) used [$^3$H]DHA to assess the levels of β-adrenoceptors in heart, lung, kidney, brain and liver tissues of sheep. It is safer to handle a tritiated ligand which can also last longer than iodinated ligands. In addition, there is no significant alteration of chemical structure of the tritiated ligand by the presence of the isotope (Williams and Lefkowitz 1978). However, a tritiated ligand has a lower specific radioactivity than an iodinated ligand, and this could be a disadvantage when the concentration of the β-adrenoceptors is low. Williams and Lefkowitz (1978) also indicated that the best data can usually be obtained in the range of 1 nM to 20 nM [$^3$H]DHA when the total volume of incubation is 150 μL, as used in this study.

Propranolol was used in this study because it is also a potent antagonist for β1- and β2- adrenoceptors. It is commonly used for nonspecific binding because it can completely displace a radioligand from the receptors (Bylund et al. 1994). It is advisable to use at least 100 times the Kd value (10 nM) of propranolol for complete displacement of DHA. The concentration of propranolol used for this study was 10 μM. Tseng et al. (1995a) used a concentration of 1 μM for studying β-adrenoceptors in sheep tissues. Terbutaline has a high affinity for β2-adrenoceptors, but the affinity for β1-adrenoceptors is about 1,000 times less (Bylund et al. 1994). In this study, 5 μM of terbutaline was used to

saturate β2-adrenoceptors, but was low enough not to affect β1-adrenoceptors. However, since the use of terbutaline was not tested with sheep tissues, it is not known whether the concentration of terbutaline chosen was enough to saturate all the binding sites for β2-adrenoceptors. If that was not the case, this could complicate the binding data.

In adition to receptor proteins, the crude plasma membrane fractions may contain varying amounts of other proteins and microsomes (ohlendieck et al. 1991). Binding of the radioligand to these non-β-adrenoceptors will contribute to the total binding. If their contributions to total and nonspecific bindings are not equal, binding to these non-β-adrenoceptors could contribute to variations in the binding data obtained. This would eventually affect the interpretation of the results. These variations are expected because different membrane fractions are not expected to have similar amounts and / or types of non-β-adrenoceptor components. Figure 6.1 shows the specific binding curve and the lines for total and nonspecific binding. The nonspecific binding is a linear regression forced through zero, and it is parallel to the straight line of total binding. The total binding curve became linear after the saturation of the specific binding sites, and from this point the total binding is expected to be 100 % nonspecific binding. Table 6.2 shows the values of nonspecific bindings obtained at the Kd (4 nM) of [$^3$H]DHA for all the tissues studied. The Kd value of 4 nM for [$^3$H]DHA as determined by Williams and Lefkowitz (1978) and Tseng et al. (1995a) was used here with the assumption that it applies to all the tissues studied. The absolute values of nonspecific binding for the heart membrane was the highest and that of the liver was the least. For all the tissues, the nonspecific binding was about 9 to 37 % of the total binding at the Kd for [$^3$H]DHA. This

indicates a wide range of variations which could complicate the binding data obtained in chapters 3 and 4. This was despite the fact that the filters were pre-soaked in 0.5 % polyethylenimine to reduce nonspecific binding. The extent of variation of the nonspecific binding is also indicated by the large coefficient of variation (Table 6.2). Mersmann and McNeel (1992) obtained nonspecific binding of about 7 to 15 % of total binding for porcine adipose tissue using a Kd value 5 nM for [$^3$H]DHA. Nonspecific binding of zero to 15 % and 15 to 40 % of total binding, using [$^3$H]DHA as radioligand (Kd = 4 nM), were reported for a variety of tissues from human, frog and rat (Williams and Lefkowitz 1978). It is obvious that the levels of nonspecific binding varies from assay to assay, from tissue to tissue, and may also depend on tissue preparation. The amount and types of proteins and other components of the plasma membrane fractions vary. These variations, in addition to the differences among replicate tubes, contribute to variation in nonspecific binding for the different tissues. Unfortunately, the extent to which the variation in nonspecific binding affected the binding data in the present study is not known. However, the range of nonspecific bindings in this study was similar to that reported by others.

Another aspect of the methodology that might have complicated the binding data and results of the Northern blot analysis is the fact that the animals were fasted for 24 hr. This was done to reduce the gut fill in order to facilitate evisceration and tissue sampling. As indicated in chapter 2, the plasma concentrations of hormones were altered after fasting the animals. It is not known whether the results of the binding study and Northern blot analysis were affected by fasting, and if so, to what extent. The 24 hr fast was common to

all treatments and therefore valid comparisons can be made among treatments. Although the preslaughter fast might have reduced the magnitude of treatment effects, those differences which were observed likely were a true reflection of the differing physiological states that existed prior to fasting. It may be that some of the trends observed in response to treatment may have reached significance if the animals had not been fasted prior to slaughter.

### 6.4 Relating the Findings to Animal Production

In ruminants, adrenergic agents, through their effects on energy and protein metabolism, are associated with increased milk production and quality, increased lean meat production and increased adaptability of animals to their environments (Jaster and Wegner 1981, Ricks et al. 1984, Kim et al. 1992). These studies were conducted by administration of β-agonists and antagonists to the animals. Activation of β-adrenoceptors by β-agonists led to an increase in average daily gain, a decrease in feed intake or both, in farm animals (Moloney et al. 1991). In chapters 3 and 4, radioligand binding assays indicated that cold exposure and feed restriction increased the densities of β1-adrenoceptors in skeletal muscle, kidney and liver tissues of sheep. Assuming the concept of 'spare receptors' where occupancy of a fraction of the receptors results in maximum biological responses (Roth and Grunfeld 1985) does not apply, the increase in β1-adrenoceptor number could potentially increase metabolic responsiveness of tissues to beta-agonists. Cold exposure is known to cause an increase in concentrations of endogenous plasma catecholamines in sheep (Christopherson et al. 1978, see also Table 6.3). These catecholamines could play a

role in cold-induced thermogenesis (Young et al. 1989) or in changes in average daily gain as observed in chapter 2. As indicated in chapter 2, cold exposure increased the average daily gain of the *ad libitum* fed group.

The whole body metabolic response was partly inhibited by non-selective β-adrenoceptor blocker, propranolol (Webster et al. 1969), thus implicating involvement of β-adrenoceptors in body metabolism. With increased catecholamine release in the cold (Table 6.3), this may partly explain the increased metabolic rate of the *ad libitum* fed group observed in chapter 2. The metabolic rates of the feed restricted groups were lower than those of the *ad libitum* fed groups, even though they had higher levels of $\beta_1$-adrenoceptors (chapters 2, 3 and 4). Considering the role of catecholamines in metabolism, the high level of β1-adrenoceptors in feed restricted groups may not be interpreted as promoting reduced energy expenditure. Other factors are likely to be responsible. However, the reduction in metabolic rates in feed restricted groups may be related to suppression of sympathetic activity (Shetty and Kurpad 1990). This adaptation to low energy intakes is likely to be important to animal survival during periods of scarce feed supplies. The reduction in metabolic rate could be economically important if it is associated with reduced maintenance cost. The present study shows that insufficient nutrients combined with increased maintenance cost in the cold could restrict the productivity of the animals. This is because the lambs in the cold-restricted (CR) group had the lowest carcass weights (chapter 2).

The ultimate goal of characterization of adrenergic receptors is to gain insight into molecular mechanisms by which adrenergic agents elicit physiological responses.

Understanding these mechanisms may result in effective approaches to manipulation of livestock production through improvement of energy and protein metabolism. This research has provided useful information on the expression and regulation of β-adrenoceptors in sheep as a step towards improved understanding of their involvement as metabolic regulators. This information could be extended to other ruminants, but with caution, due to possible species variation. However, ligand affinity for a receptor does not always predict biological function, and *in vitro* activity may not mimic the activity *in vivo* (Mersmann 1995). Therefore, the interpretation of the results may be limited due to the facts that there are additional factors that are unknown or have not been considered.

In domestic animals, most of the functions of β-adrenoceptors have been established through administration of beta- agonists and antagonists. Variation in responses has also been known to be due to divergent distribution of β-adrenoceptor subtypes, and tissue and species differences in the structures of these receptors (Mersmann 1995). Therefore, it is important to understand the distribution of β-adrenoceptors in tissues in order to estimate how beta- agonists and antagonists would affect the different tissues. This research has provided some information on the distribution of β-adrenoceptors in tissues of sheep. Although the present study may be different from the physiological responses involving interaction of catecholamines with β-adrenoceptors or an *in vivo* study of receptors using radionuclide scan as suggested by Motulsky and Insel (1987), this research has provided useful information on the expression and regulation of β-adrenoceptors in ruminants. This research represents another step towards improved understanding of the role of

catecholamines and adrenergic receptors in relation to acute and chronic cold exposure on high and low planes of nutrition, as summarized in Table 6.3.

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**Table 6.1** Ratios[Z] of β1-adrenoceptor densities to β1-adrenoceptor mRNA densities in tissues of sheep

| | WA | WR | CA | CR |
|---|---|---|---|---|
| *Biceps femoris* | 20.01aq | 13.67br | 15.42bq | 8.83cq |
| *Semitendinosus* | 33.33an | 16.85cq | 26.83bn | 13.17dp |
| *Gastrocnemius* | 17.47br | 25.93an | 21.05ap | 27.34am |
| Heart | 15.42br | 20.64ap | 14.41cq | 17.36bn |
| Kidney | 25.20ap | 17.17bq | 24.39an | 9.80cq |
| Liver | 77.78am | 39.47cm | 44.44bm | 29.23dm |

[Z]Ratios are in fmol..mg$^{-1}$ proteins.% volume$^{-1}$.

a, b, c, d Values within row with different letters are different ($P < 0.05$).

m, n, p, q Values within column with different letters are different ($P < 0.05$)

**Table 6.2** Nonspecific binding data[Z] for binding of tritiated dihydroalprenolol ([$^3$H]DHA) to crude plasma membranes isolated from sheep tissues.

| | Mean | SD | % of Total | CV[Y] |
|---|---|---|---|---|
| *Biceps femoris* | 10.35 | 4.27 | 9 - 23 | 41.3 |
| *Gastrocnemius* | 3.57 | 2.54 | 5 - 12 | 71.2 |
| *Semitendinosus* | 10.05 | 3.71 | 15 - 27 | 36.9 |
| Heart | 43.7 | 11.02 | 11 - 29 | 25.2 |
| Kidney | 14.7 | 5.17 | 21 - 37 | 35.2 |
| Liver | 4.3 | 2.79 | 13 - 28 | 64.9 |

[Z] Mean values are in fmol.mg$^{-1}$ protein (N = 24). Values were obtained at the Kd (4.0 nM) for DHA. Incubation was carried out at 25 °C for 30 min.

[Y] CV indicates coefficient of variation (SD/mean X 100 %).

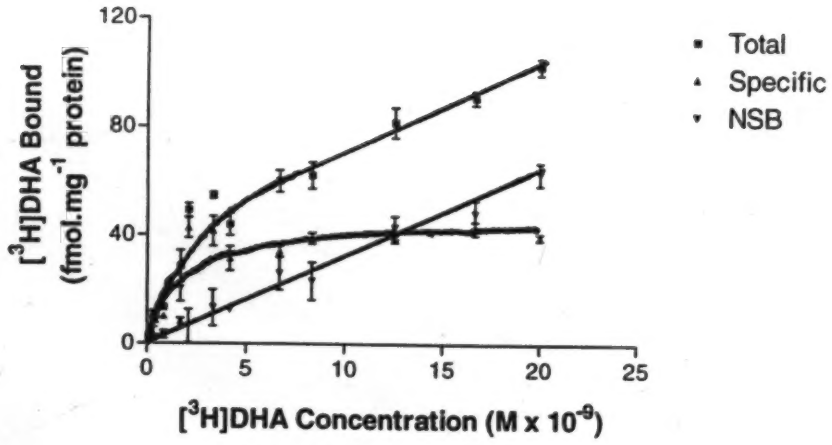
**Table 6.3** The role of catecholamines and adrenergic receptors in ruminants in relation to acute and chronic cold exposure on high and low planes of nutrition[Z]

| Cold Exposure | Acute Cold | | Chronic Cold | |
|---|---|---|---|---|
| Plane of Nutrition | Low | High | Low | High |
| Plasma Epinephrine (E)[1, 2, 3, 4] | + + + | | + + | + + |
| Plasma Norepinephrine (NE)[1, 2, 3, 4] | + + + + | | + + + | + + + |
| Responsiveness to E and NE[3, 5, 6] | | | | |
| Heat production | | + + | | = |
| Skin temperature | | | | = |
| Plasma glucose | | + + | + | + + |
| Plasma free fatty acids | | | + + | + |
| Plasma total amino acids | | + + | | + / - |
| Plasma glycerol | | + | | |
| Rumen motility | | | | + / - |
| Responsiveness to β-Blockers[3,5,8] | | | | |
| Heat production | | + / - / = | | - |
| Rectal temperature | | + | | |
| Skin temperature | | = | | |
| Plasma glucose | | = | | = |
| Plasma total amino acids | | + | | = |
| Plasma glycerol | | = | | = |
| β1-adrenoceptor density[9] | | | + / - | = |
| β1-adrenoceptor mRNA density[9] | | | + / - | = |
| β2-adrenoceptor density[9] | | | = | = |

[Z] + increased concentration or responsiveness; - decreased concentration or responsiveness; = similar concentration or responsiveness, compared to thermoneutral control (approximate magnitude of increase or decrease indicated by number of signs).

[1]Christopherson et al. (1978); [2]Graham et al. (1981); [3]Harman (1992); [4]Thompson et al. (1978); [5]Webster et al. (1969); [6]Graham and Phillips (1981); [7]Graham et al. (1982); [8]Miaron (1994); [9]Present study

**Figure 6.1** Total binding, nonspecific binding (NSB) and specific binding of [$^3$H]dihydroalprenolol ([$^3$H]DHA) to crude plasma membrane fraction isolated from *biceps femoris* muscle (each point represents the mean ± SEM of two replicate tubes in the single assay for one animal).



## Appendix 1

**Table A1.1** Weights of wether lambs[Z] in Kg[Y]

| Period | A11 | A13 | A23 | A34 | A37 | A38 | B5 | B7 | B22 | B28 | B35 | B36 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 42 | 40 | 37 | 40 | 28 | 33 | 41 | 36 | 44 | 36 | 32 | 32 |
| I | 50 | 53 | 52 | 49 | 40 | 42 | 44 | 39 | 46 | 40 | 37 | 36 |
| II | 57 | 64 | 61 | 54 | 47 | 50 | 52 | 46 | 56 | 51 | 41 | 42 |
| III | 61 | 72 | 70 | 60 | 56 | 58 | 57 | 49 | 62 | 54 | 43 | 45 |

[Z]All the A's are in WA group and all B's are in WR group.

[Y]weights are at the end of the period.

**Table A1.2** Weights of wether lambs[Z] in Kg[Y]

| Period | C4 | C6 | C16 | C24 | C27 | C31 | D9 | D15 | D30 | D32 | D33 | D39 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 39 | 38 | 41 | 37 | 34 | 36 | 39 | 40 | 38 | 35 | 29 | 37 |
| I | 45 | 50 | 50 | 41 | 44 | 40 | 40 | 41 | 40 | 39 | 32 | 39 |
| II | 61 | 60 | 64 | 56 | 59 | 45 | 47 | 44 | 46 | 43 | 37 | 39 |
| III | 72 | 71 | 74 | 62 | 70 | 54 | 50 | 46 | 52 | 46 | 41 | 43 |

[Z]All the C's are in CA group and all D's are in CR group.

[Y]weights are at the end of the period.

## Appendix 2

**Table A2.1** Values[Z,Y] of coefficient of variation for beta-1 adrenoceptors in tissues of sheep

| Tissue | WA | WR | CA | CR |
|---|---|---|---|---|
| *Biceps femoris* | 18.9 | 29.3 | 26.1 | 34.6 |
| *Semitendinosus* | 28.7 | 36.5 | 20.3 | 23.2 |
| *Gastrocnemius* | 12.4 | 18.0 | 25.4 | 21.3 |
| Heart | 32.2 | 18.5 | 16.7 | 24.9 |
| Kidney | 16.1 | 21.2 | 24.4 | 11.2 |
| Liver | 27.6 | 16.4 | 21.1 | 23.1 |

[Z]All values are in percent.

[Y]Coefficient of variation (standard deviation / mean X 100 %) values were calculated using the GLM procedure of SAS (SAS 1990).

**Table A2.2** Values[Z,Y] of coefficient of variation for beta-1 adrenoceptor mRNA in tissues of sheep

| Tissue | WA | WR | CA | CR |
|---|---|---|---|---|
| *Biceps femoris* | 40.1 | 18.1 | 16.2 | 14.7 |
| *Semitendinosus* | 19.9 | 21.6 | 26.9 | 20.5 |
| *Gastrocnemius* | 68.2 | 42.2 | 43.4 | 44.6 |
| Heart | 18.0 | 20.0 | 26.8 | 24.5 |
| Kidney | 18.8 | 7.5 | 8.5 | 22.3 |
| Liver | 56.6 | 58.2 | 21.2 | 38.0 |

[Z]All values are in percent.

[Y]Coefficient of variation (standard deviation / mean X 100 %) values were calculated using the GLM procedure of SAS (SAS 1990).

**Table A2.3** Variation in efficiency of growth and metabolic rate[Z,Y]

| | WA | WR | CA | CR |
|---|---|---|---|---|
| Period 1 | | | | |
| Average daily gain | 23.6 | 29.9 | 44.6 | 52.8 |
| Feed efficiency | 24.5 | 35.4 | 42.4 | 50.8 |
| Metabolic rate | 15.4 | 18.5 | 26.0 | 20.4 |
| Period 3 | | | | |
| Average daily gain | 26.9 | 40.9 | 20.3 | 37.1 |
| Feed efficiency | 23.6 | 31.9 | 18.6 | 36.1 |
| Metabolic rate | 6.3 | 9.5 | 21.5 | 34.6 |

[Z]All values are in percent.

[Y]Coefficient of variation (standard deviation/mean X 100 %) values were calculated using the GLM procedure of SAS (SAS 1990).

## Appendix 3

**Table A3.1** Composition of experimental diet (Dehydrated Alfalfa pellets)

| Nutrients, Minerals or Vitamins | Amount |
|---|---|
| Protein (%) | 17.00 |
| Total Digestible nutrients (%) | 66.60 |
| Crude Fibre (%) | 25.00 |
| Acid detergent fibre (%) | 34.00 |
| Calcium (%) | 1.50 |
| Phosphorus (%) | 0.22 |
| Potassium (%) | 2.39 |
| Copper ($mg.Kg^{-1}$) | 11.00 |
| Manganese ($mg.Kg^{-1}$) | 34.00 |
| Zinc ($mg.Kg^{-1}$) | 21.00 |
| Magnesium (%) | 0.32 |
| Selenium ($mg.Kg^{-1}$) | 0.37 |
| Carotene ($mg.Kg^{-1}$) | 180 |
| Xanthophyll ($mg.Kg^{-1}$) | 292 |
| Vitamin E ($mg.Kg^{-1}$) | 123 |
| Niacin ($mg.Kg^{-1}$) | 44 |
| Thiamine ($mg.Kg^{-1}$) | 4.10 |
| Choline ($mg.Kg^{-1}$) | 1,656 |
| Lysine (% of protein) | 4.90 |
| Threonine (% of protein) | 4.10 |
| Methionine & Cystine (% of protein) | 3.20 |
| Tryptophan (% of protein) | 2.00 |